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Changing face of irritable bowel syndrome

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Abstract

Recent years have witnessed tremendous progress in our understanding of irritable bowel syndrome (IBS). It is evident that this is a truly global disease associated with significant symptoms and impairments in personal and social functioning for afflicted individuals. Advances in our understanding of gut flora-mucosal interactions, the enteric nervous system and the brain-gut axis have led to substantial progress in the pathogenesis of symptoms in IBS and have provided some hints towards the basic etiology of this disorder, in some subpopulations, at the very least. We look forward to a time when therapy will be addressed to pathophysiology and perhaps, even to primary etiology. In the meantime, a model based on a primary role for intestinal inflammation serves to integrate the various strands, which contribute to the presentation of IBS

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Key words: Irritable bowel syndrome; Functional gastrointestinal disease; Intestinal inflammation

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EPIDEMIOLOGY

In recent years, the true prevalence of IBS has been documented in many parts of the world. What is truly remarkable is how common IBS is, no matter where you look! It is absolutely clear, for example, that IBS is a common disorder, not only in North America and Western Europe but throughout Asia and Latin America and even in parts of Africa^[1]. However, caution needs to be exerted in the interpretation of such studies. Typically,

community or hospital-based surveys of IBS prevalence have utilized some iteration of Rome or Manning criteria as their diagnostic instrument; whether these diagnostic tools, developed in the West, are equally valid in emerging nations, where confusion with symptoms related to chronic parasitic infestations, for example, may be an issue. Clearly, we have much to learn from the epidemiology and natural history of IBS or IBS-like symptoms in this context.

DIAGNOSIS

Diagnostic confusion has also emerged as an issue in the West. Here, still the debate continues regarding potential overlap between IBS, IBD and celiac sprue^[2]. Do reported instances of celiac sprue among patients with “typical” IBS, or the occurrence of IBS-type symptoms among IBD patients in apparent remission, reflect a true association between these disorders and, thereby, the effects of low grade inflammation on enteric nerve and muscle function, or does such apparent overlap simply serve to emphasize the non-specificity of many gastrointestinal symptoms? Explaining it simply, the gut has a limited symptomatic repertoire which may not allow us to differentiate between those complaints which are consequent upon continuing (but otherwise undetected) inflammation in IBD or in the non-compliant celiac and those which arise from a functional disorder, *per se*. Progress in this contentious area must await readily applicable measures of disease activity which are sufficiently sensitive and accurate to provide a true definition of remission.

In the meantime, how should the clinician interpret these dilemmas? It is evident, that the majority of celiacs now present later in life and usually with vague and non-specific gastrointestinal symptomatology; celiac disease must, therefore, be considered in all new IBS patients, especially in areas of high prevalence and regardless of the nature of presenting symptoms^[3].

ASSOCIATED DISEASES

Over the years, IBS has been associated with a wide variety of intestinal and extra-intestinal symptoms and syndromes. Recent community surveys have confirmed how frequently IBS, functional dyspepsia (FD) and gastroesophageal reflux disease (and non-erosive reflux disease (NERD), in particular) overlap; a phenomenon that may complicate clinical trials as well as diagnostic and therapeutic strategies. My own belief is that we should be “lumpers” and not “splitters” here; I contend that efforts to separate IBS from FD and NERD are clinically unrealistic and

unhelpful. IBS has also been associated with a variety of psychological disorders; here, in contrast, the evidence for a true association is less firm, more recent analyses suggest that the occurrence of such symptomatology in IBS is largely the preserve of those who seek further referral alone and is not a feature of IBS in the community. Psychopathology should be viewed, therefore, not as a fundamental prerequisite for the development of IBS, but, rather, as a co-factor which, if present, will modify the individual's response to IBS symptomatology. IBS patients commonly complain of fatigue and tiredness; these appear to be real entities in IBS, yet have been scarcely acknowledged in the assessment of IBS activity or response to therapy. Urinary and gynecological symptoms are also common; the basis for these associations is less clear. Aware of the prominence of smooth muscle hyper-reactivity in both conditions, parallels have been drawn between IBS and asthma; whether these conditions are linked remains to be defined.

PATHOPHYSIOLOGY

Genetic factors

While IBS patients commonly give a positive family history, the relative roles of "nature" and "nurture" in this intra-familial aggregation of functional disorders have received little attention. For example, in a recent community survey almost 20% of IBS sufferers reported abdominal symptoms in a first degree relative; a relative risk of 2.5^[4]. Whether this association reflects reporting bias, shared environmental factors or a true genetic basis has been addressed in two recent twin studies which both identified a genetic component to IBS^[5,6]. This is not the whole answer by any means; thus in the study by Levy and colleagues, while the concordance for IBS was twice as high in monozygotic than in dizygotic twins (15.2% *vs* 6.7%), a history of IBS in a parent was a more potent predictor of IBS in a twin than was the presence of IBS in the other twin^[6]. These findings suggest a relatively minor role for genetic factors in the basic pathogenesis of IBS. Genetic factors may, however, influence disease expression and therapeutic response, as evidenced by recent studies of G-protein subunit, IL-10, CCK-1 receptor, alpha 2 adrenoceptor and serotonin transporter genotypes among IBS patients^[7-11]. These are complex studies but may pave the way for real progress in our understanding of the true diversity of IBS^[12].

Gastrointestinal motor dysfunction

Dysmotility has long been considered a major factor in the pathophysiology of IBS, as indicated by the use, in the past, of such terms as the "spastic colon" to describe what is now referred to as IBS. Accordingly, it was suggested that gut spasm or other abnormal contractile activities led to the development of symptoms in IBS. There are, indeed, several reports of abnormal motor patterns in many parts of the gastrointestinal tract in IBS. The specificity of many of these abnormalities for IBS is, however, unclear^[13]. In contrast, and of particular interest, are very recent observations on the handling of gas by the intestine in IBS^[14,15]. Whereas gas infused into

the small intestine was rapidly evacuated through the gut in normal volunteers, a similar infusion resulted in gas retention, symptoms and an increase in abdominal girth in IBS patients^[14]; all reversible by administration of a prokinetic agent^[15]. Distension, often the most distressing "gas"-related symptom in IBS, has, until recently, been assumed to represent a disturbance of perception, as apparently objective tests of abdominal volume failed to detect any increase in IBS^[16]. This assumption has now been questioned^[17] and it may well come to pass that more detailed studies of changes in distension over time^[18] may detect significant diurnal variations in girth in IBS. While the balance of evidence suggests that intestinal gas production is not abnormal in IBS, one can visualize how relatively local changes in the gas content could lead to symptoms, given the aforementioned intrinsic abnormality of gas transport^[19] and the hypersensitivity to intraluminal gas that are known to occur in IBS^[20].

Visceral hypersensitivity and hyperalgesia

Recently, there has been considerable interest in these phenomena, not only in IBS, but also in functional disorders, in general^[7]. The phenomenon of visceral hypersensitivity, to distention and other intra-luminal stimuli, appears to be common in patients with non-cardiac pain, FD and the irritable bowel, alike. Recently, it has been suggested that visceral hyperalgesia, the phenomenon whereby stimuli normally not experienced as painful become so, is highly specific for IBS^[21]. Visceral hypersensitivity, visceral hyperalgesia and viscerosomatic referral (the phenomenon whereby stimuli are referred over wide areas) have, indeed, been confirmed, in IBS, in more recent studies, using a variety of methodologies and under controlled experimental conditions^[22]. While visceral hyperalgesia has been postulated as being highly specific for IBS, it alone or in association with other manifestations of hypersensitivity cannot explain all of IBS; even the most celebrated enthusiasts for the sensory hypothesis concede that sensation is normal in some patients.

There are several possible anatomical locations for sensory abnormalities in IBS, ranging from sensory receptors on the gut wall, primary sensory afferent neurons, to the spinal cord and the brain itself. Research in this area in man is notoriously difficult; however, advances in functional brain imaging provided by such techniques as cerebral evoked potentials (CEP), positron emission tomography (PET), magnetoencephalography (MEG) and functional magnetic resonance imaging (fMRI), have provided insights into the brain's response to visceral stimuli. These and other studies have advanced the concept of an abnormal (or hypervigilant) central nervous system (CNS), in IBS, which records an exaggerated, inappropriate or aberrant perception of visceral events^[23]. Other pieces of evidence support this concept. These include the conscious perception, by IBS patients, of intestinal motor events which are usually sub-conscious and evidence of abnormal psycho-neuro-hormonal responses, often implicating an abnormal hypothalamo-pituitary axis (HPA).

Motility and sensation may not be the most fundamental

causes of IBS; it is clear, however, that these phenomena play a significant role in symptom generation.

Infection, inflammation, immunity and IBS

It may come as a real surprise to many to hear that infection and inflammation are now seen as potential factors in the etiology of IBS. With regard to infection, we are now beginning to see the real data to directly support the concept of post-infective or post-dysenteric IBS.

Infection and IBS First reported by McKendrick and Read^[24], the occurrence of IBS following bacteriologically-confirmed gastroenteritis has now been documented in several studies^[25-30]. The risk of developing IBS following an episode of gastroenteritis is in the order of 4%-23%, with females, those with a more severe initial illness and pre-morbid psychopathology being most at risk^[25,26,28,30]. One of these studies went on to establish a direct link between prior exposure to an infectious agent, persisting low-grade inflammation and IBS^[28]. In this study, an increase in the number of chronic inflammatory cells in the rectal mucosa was seen only among those exposed patients who had developed IBS. Others have demonstrated a persisting increase in rectal mucosal enteroendocrine cells, T lymphocytes and gut permeability in patients with post-dysenteric IBS^[29,30]. Post-infectious IBS may explain only a minority of cases of IBS but does represent a clear link between exposure to an environmental agent, inflammation and IBS, in predisposed individuals^[31].

Inflammation and IBS Direct and compelling evidence was first provided by Chadwick and colleagues for a role of inflammation in IBS, in general. They evaluated 77 IBS patients of whom 55% would be considered as diarrhea predominant; none had a confirmed infectious origin for their IBS^[32]. All had colonic biopsies taken for conventional histology and immunohistology. Thirty-eight had normal histology, 31 demonstrated microscopic inflammation and 8 fulfilled the criteria for lymphocytic colitis. However, in the group with "normal" histology, immunohistology revealed increased intraepithelial lymphocytes as well as an increase in CD3+ and CD25+ cells in the lamina propria; all, therefore, showed evidence of immune activation. These features were even more evident in the microscopic inflammation group who, in addition, revealed increased neutrophils, mast cells and natural killer cells. All of these aforementioned immunopathological abnormalities were most evident in the lymphocytic colitis group who, alone, also demonstrated HLA-DR staining in crypts and increased CD8+ cells in the lamina propria. Interestingly, taking the group of IBS patients as a whole, CD3+ cell number was higher among those with diarrhea than among alternators or those with predominant constipation. In contrast, in the non-inflamed IBS group the presence of mast cells was a predictor of constipation. Surprisingly, given the aforementioned description of a direct relationship between symptoms and chronic inflammation among patients with post-infectious IBS, these authors did not find an association between either the nature of disease onset or disease duration and immunological findings. In an accompanying editorial, Collins suggested that the increased presence of CD25+ cells may have indicated "auto- or exogenous antigen challenge in these patients,

and that the CD25+ cells are preventing the progression to a more florid inflammatory response"^[33]. That IBS patients may be predisposed to an, *albeit* contained, inflammatory response to luminal triggers is also supported by the finding, of Gonsalkorale and colleagues, of a reduced frequency of the high-producer phenotype for the anti-inflammatory cytokine interleukin-10 (IL-10) among IBS patients^[9]. A direct linkage between immune activation and symptoms has been provided by the work of Barbara and colleagues who demonstrated, not only an increased prevalence of mast cell degranulation in the colon in IBS, but also a direct correlation between the proximity of mast cells to neuronal elements and pain severity^[34].

While the inflammatory hypothesis in IBS is in its infancy, there is already some evidence for the extension of the inflammatory process beyond the confines of the mucosal compartment. Tornblom and colleagues addressed this issue in ten patients with severe IBS by examining full-thickness jejunal biopsies obtained at laparoscopy^[35]. In nine, they found low-grade infiltration of lymphocytes in the myenteric plexus; four of these had an associated increase in intraepithelial lymphocytes and six demonstrated evidence of neuronal degeneration. Nine patients had longitudinal muscle hypertrophy and seven had abnormalities in the number and size of interstitial cells of Cajal. Interestingly, three of their patients reported an acute onset of their IBS; in two, possibly precipitated by gastroenteritis. The finding of intraepithelial lymphocytosis is consistent with the reports of Chadwick and colleagues^[32], in the colon and of Wahnschaffe and colleagues, in the duodenum^[36]. Most recently, in a group of 78 unselected IBS patients, we demonstrated, in peripheral blood mononuclear cells, an alteration in the ratio between the cytokines IL10 and IL12 which became skewed towards a Th1, pro-inflammatory profile^[37].

With regard to the pathophysiology of the mucosal inflammatory changes, Spiller proposed that these changes could represent a response to an initial bacterial infection among individuals who are rendered susceptible by a relative deficiency of anti-inflammatory cytokines^[38]. Alternately, could this low-grade inflammation represent either an abnormal reaction to the normal flora or a contained response to qualitative or quantitative changes in the intrinsic flora? Whether IBS is accompanied by quantitative or qualitative changes in the bacterial flora of the small or large intestine remains a contentious issue; while some have described bacterial overgrowth in the small intestine^[39,40] and qualitative alterations in the fecal flora^[41,43] and increased bacterial fermentation^[44], in IBS, others have failed to replicate these findings^[45]. The description of efficacy for certain probiotics, and *bifidobacterium*, in particular, in IBS^[37] could also support a role of gut flora-mucosal interaction in IBS^[46]. Bacterial overgrowth could also explain some of the proposed overlap between IBS and celiac sprue^[47].

MANAGEMENT

Many IBS patients relate the onset of symptoms to intake of food and often incriminate specific food items. However, the role of food intolerance or food allergy in

IBS has remained undefined. While most would agree that there is scant evidence for classical food allergy in IBS, Whorwell and colleagues suggest that testing for food intolerance, utilizing IgG antibodies, can lead to a successful dietary modification regime^[48].

In recent years, much interest has been generated by serotonin and the potential role of serotonergic drugs in IBS^[49]. Tegaserod, a 5HT₄ agonist, is effective in the therapy of female patients with constipation-predominant IBS and has demonstrated efficacy against some previously "resistant" symptoms, such as bloating^[50,51]. Alosetron, a 5HT₃ agonist, is effective in females with diarrhea-predominant IBS, but its prescription is now limited due to reports on ischemic colitis^[52]. Cilansetron, a 5HT₃ agonist, is effective in both males and females with diarrhea-predominant IBS^[53]; here the specter of ischemic colitis has again become an issue with regulators, in the US. Indeed, ischemic colitis has become an issue for all of these agents, though it appears that many reports of association probably reflect diagnostic confusion *ab initio* between IBS and ischemic colitis rather than an effect of serotonergic agents, *per se*^[54].

Given the explosion that has occurred in our understanding of the enteric nervous system, and of the pathways that link it to the CNS, it should come as no surprise that many agonists and antagonists of other putative neurotransmitters and neuromodulators are under study in IBS and related disorders.

Given the potential role of infection and inflammation in at least some instances of IBS, efforts have been made to address this aspect of pathophysiology in IBS. In this regard, a probiotic, bifidobacterium infantis has proved to be a very successful agent in unselected IBS patients^[37]. Clearly, this is an area of increasing interest.

CONCLUSIONS

Our understanding of IBS has come a long way. This is a global disease associated with significant symptoms and impairments in personal and social functioning for afflicted individuals. Advances in our understanding of gut flora-mucosal interactions, the enteric nervous system and the brain-gut axis have led to substantial progress in the pathogenesis of symptoms in IBS and have provided some hints towards the basic etiology of this disorder, in some subpopulations, at the very least. We look forward to a time when therapy will be addressed to pathophysiology and, perhaps, even to primary etiology. In the meantime, as illustrated in Figure 1, I would suggest that a model based on a primary role for intestinal inflammation serves to integrate the various strands which contribute to the presentation of IBS.

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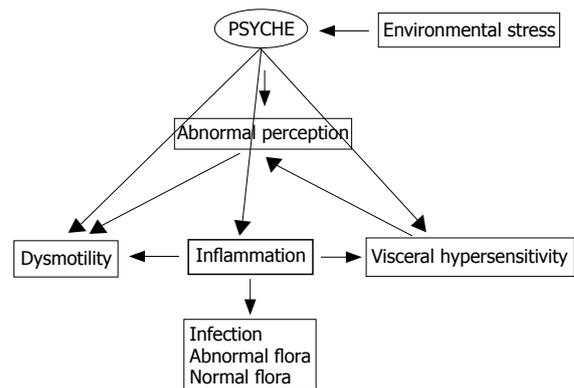


Figure 1 Interactions between the gut, the brain and the external and internal environments in IBS; the inflammation hypothesis (a personal view). Bacterial or viral infection, a disturbed flora or an abnormal response to a normal flora leads to mucosal inflammation which in turn can disrupt motility and augment visceral sensation. Centrally, perception is abnormal, thereby, contributing to symptom development. Central output can in turn influence motor events in the periphery. While not central to causation, psychological factors, either spontaneously or in response to environmental stressors, can influence motor and sensory events and immune activity in the gut.

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EDITORIAL

***Helicobacter pylori* virulence factors in duodenal ulceration: A primary cause or a secondary infection causing chronicity**

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Abstract

Reports from countries with a high prevalence of *Helicobacter pylori* (*H pylori*) infection do not show a proportionately high prevalence of duodenal ulceration, suggesting the possibility that *H pylori* cannot be a primary cause of duodenal ulceration. It has been mooted that this discrepancy might be explained by variations in the prevalence of virulence factors in different populations. The aim of this paper is to determine whether the published literature gives support to this possibility. The relevant literature was reviewed and analyzed separately for countries with a high and low prevalence of *H pylori* infection and virulence factors. Although virulent strains of *H pylori* were significantly more often present in patients with duodenal ulcer than without the disease in countries with a low prevalence of *H pylori* infection in the population, there was no difference in the prevalence of virulence factors between duodenal ulcer, non-ulcer dyspepsia or normal subjects in many countries, where the prevalence of both *H pylori* infection and of virulence factors was high. In these countries, the presence of virulence factors was not predictive the clinical outcome. To explain the association between virulence factors and duodenal ulcer in countries where *H pylori* prevalence is low, only two papers were found that give little support to the usual model proposed, namely that organisms with the virulence factors are more likely than those without them to initiate a duodenal ulcer. We offer an alternative hypothesis that suggests virulence factors are more likely to interfere with the healing of a previously produced ulcer. The presence of virulence factors only correlates with the prevalence of duodenal ulcer in countries where the prevalence of *H pylori* is low. There is very little evidence that virulence factors initiate duodenal ulceration, but they may be related to failure of the ulcer to heal.

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HELICOBACTER PYLORI VIRULENCE FACTORS AND DUODENAL ULCERATION

Following Warren and Marshall's historic paper in 1984^[1], evidence for an association between duodenal ulceration and *Helicobacter pylori* (*H pylori*) infection has been strengthened. Earlier publications, however, were from developed countries, where the overall prevalence of *H pylori* infection was between 40% and 60%. The difficult problem that remained was why everyone with *H pylori* infection did not develop duodenal ulceration. This problem was increased by the reporting of the "African enigma" from the savannah regions of the West Coast of Africa^[2,3], where the prevalence of *H pylori* infection was much higher (> 90%), but the prevalence of duodenal ulceration was relatively low. This was followed by an increasing number of reports from other countries, where a high prevalence of *H pylori* infection did not correlate with a high prevalence of duodenal ulceration (Africa^[4-8], India^[9-11], China^[12,13], Japan^[14,15], Korea^[16], Peru^[17], Iran^[18], Vietnam^[4]).

When it was later reported that some strains of *H pylori* were more virulent than others, this seemed a possible explanation of the paradox. In the more developed countries, the virulent factors, cagA (cytotoxin associated antigen) and vacA (vacuolating factor), were present in between 40% and 60% of *H pylori* strains, and it was suggested that these strains might prove to be the causal factors in duodenal ulceration and account for the discrepancies^[19-24].

There is no doubt about the association of these factors with duodenal ulceration in countries, where the overall prevalence of *H pylori* infection and virulence factors is relatively low, compared with countries where it is high. However, an increasing number of reports from countries, where *H pylori* infection is almost ubiquitous (70-90+%) and 77%-88% of the strains carry the viru-

lence factors *cagA* and *vacA*, have shown no relationship between these factors and clinical outcome (South Africa^[6], India^[25,26], China^[27-31], Japan^[32-43], Korea^[44-47], China Taiwan^[48], Thailand^[49], Sudan^[50], Turkey^[51-53], Nigeria^[54,55], Sri Lanka^[56], Bangladesh^[57], Serbia Montenegro^[58], Estonia^[59], Brazil^[60], Singapore^[61,62], Mexico^[63]). A few similar reports have also come from countries with a low prevalence (Germany^[45,64], France^[65,66], Finland^[67], UK^[68,69], USA^[42]).

Most *cagA* positive strains also carry the *vacA* gene. When present, the *cagA* gene secretes the toxic CagA protein, but not all *vacA* strains secrete a toxigenic protein. There are different allelic types of *vacA*, the types *vacAs1* and *vacAs1m1* are toxic and strongly associated with duodenal ulceration, mostly in countries with a relatively low prevalence of *H pylori* infection. Once again, however, reports from countries with a high prevalence of these factors show no link between the presence of *vacAs1*^[6,26,29,38,44,47-49,56,57,60,70] or *vacAs1m1*^[25,26,29,41,44,52,55,59,65,70] and clinical outcome.

Other virulence markers have been reported: *iceA1* gene [induced by contact with gastric epithelium] and *babA2* gene (blood group antigen binding adhesin), which binds to Lewis B present on gastric epithelial cells, show an association with duodenal ulceration in countries, where the prevalence of *H pylori* and these strains is low. However, reports from many countries with a high prevalence of *H pylori* and these virulence markers again show that in these areas they are not predictive of the clinical outcome (*iceA1*^[6,26,31,41,45,48,52,55,57,61,62,71-73], *babA2*^[6,32,39,41,42,47,61]).

Thus, there remains the anomaly that, although duodenal ulceration is strongly associated with *H pylori* infection and certain virulence factors in countries with a relatively low prevalence of both *H pylori* infection and virulence factors, this association disappears in many countries^[74] where these prevalences are high, and where *H pylori* infection and virulence factors do not predict clinical outcome. This casts doubt upon whether *H pylori* initiates duodenal ulcer. This doubt is strongly supported by the finding that most patients with a short history^[75] or all with less than 6 month's history^[76] of duodenal ulcer symptoms were uninfected with *H pylori*.

Nonetheless the importance of *H pylori* infection and virulence factors cannot be dismissed. There is no doubt that the eradication of *H pylori* infection leads to healing of duodenal ulceration and the risk of recurrence is greatly reduced. There is also no doubt about the strong association of *H pylori* and virulence factors with duodenal ulceration in countries where the overall prevalence of *H pylori* infection is relatively low.

The tendency for *H pylori* to be absent in the early case suggests that the organism is not the primary cause producing duodenal ulcer. The evidence that the chronic course of healing→recurrence→etc. of the typical chronic duodenal ulcer is converted in most cases into stable healing by eradicating *H pylori* suggests that the organism, when present, interferes with the healing process.

There remains the question why the virulence factors are related to the presence of duodenal ulceration in the countries with a low prevalence of *H pylori* infection. It is possible that colonization of nearby areas of antral epithelium or of gastric metaplasia in the duodenum by

H pylori leads to the local release of toxins that produces the duodenal ulcer. However, this straightforward model has not been substantiated. The toxins concerned have been demonstrated and their toxic effects are determined mostly by their interaction with gastric epithelium^[19] and there are only two papers reporting about the damage caused by toxins to duodenal mucosa. One paper^[77] reports about the prevention of healing of mechanically abraded human duodenal epithelium *in vitro* by strains of wild *H pylori*, particularly those carrying the *vacA* gene, and also by supernatant fluid containing the *vacA* cytotoxin. The other paper^[78] reports about increased duodenal mucosal permeability, when exposed to *H pylori* culture fluid in rats. It must be emphasized that neither paper reports about the *initiation* of ulceration.

As an alternative explanation, we advance the following more complicated model which we have partly suggested before^[11,76]. *H pylori* is killed by excess acid^[79,80]. In countries, where the overall prevalence of *H pylori* infection is low, duodenal ulcer patients initially may be free from *H pylori* infection because of their high acid output. In the early stages of ulceration, many subjects, prior to seeking definitive treatment, control their symptoms with antacids, some including H2 antagonists, which are available without prescription. This reduces the defense against infection with the organism in patients who have hitherto been resistant (since they start *H pylori* negative). This partial reduction in resistance can be overcome by virulent, but not by non-virulent strains, so there is an association between the virulent strains and the chronic ulcer patients, most of whom have become *H pylori*-positive for the organism by 6 months time^[75,76]. The high baseline of infection with virulent strains, in the countries with a high prevalence, obscures this effect.

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EDITORIAL

Causal role of *Helicobacter pylori* infection and eradication therapy in gastric carcinogenesis

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Abstract

Many epidemiological reports indicate that *Helicobacter pylori* (*H pylori*) infection plays an important role in gastric carcinogenesis. Several genetic and epigenetic alterations contribute to the initiation, promotion, and progression of the cancer cells in a multi-step manner. *H pylori* is known to induce chronic inflammation in the gastric mucosa. Its products, including superoxides, participate in the DNA damage followed by initiation, and the inflammation-derived cytokines and growth factors contribute to the promotion of gastric carcinogenesis. By eradicating *H pylori*, gastric inflammation can be cured; the therapy diminishes the levels not only of inflammatory cell infiltration, but also atrophy/intestinal metaplasia in part. A randomized controlled trial revealed that the eradication therapy diminished the gastric cancer prevalence in cases without pre-cancerous conditions. In addition, recent epidemiological studies from Japanese groups demonstrated that the development of gastric cancer, especially of the intestinal type, was decreased by successful eradication therapy, although these were designed in a non-randomized manner. However, it should be mentioned that endoscopic detection is the only way to evaluate the degree of gastric carcinogenesis. We have reported that the endoscopic and histological morphologies could be modified by eradication therapy and it might contribute to the prevalence of gastric cancer development. Considering the biological nature of cancer cell proliferation, it is considered that a sufficiently long-term follow-up would be essential to discuss the anticancer effect of eradication therapy.

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Key words: *H pylori*; Eradication; Gastritis; Gastric neoplasm; Endoscopy

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INTRODUCTION

Helicobacter pylori (*H pylori*), a Gram-negative bacillus discovered in 1983, is the most popular pathogenic bacteria in the world. Approximately half of the population has *H pylori* infection worldwide, and the prevalence is thought to be 80-90% in developing countries and 30-50% in developed countries^[1]. Many studies clarified the implication of the bacteria as the cause of many gastroduodenal diseases, including histological chronic gastritis, peptic ulcer, and mucosal-associated lymphoid tissue (MALT) lymphoma^[2,3].

In particular, histological chronic atrophic gastritis is an important disease for gastric carcinogenesis. Two main causes for the promotion of atrophic gastritis are (1) *H pylori* and (2) autoimmune factors such as antiparietal cell antibody. Although these two factors work synergistically in the promotion of atrophic gastritis^[4,5], autoimmune gastritis is a rare disease and most atrophic gastritis is thought to be induced by *H pylori* infection in East-Asian countries such as in Japan, China, and Korea^[6]. Therefore, it is likely that *H pylori* is the most important factor for mucosal atrophy and we have confirmed this hypothesis in the Japanese population^[7].

Gastric cancer is the second most common cancer (next to lung cancer) in the world and the number of newly diagnosed cases was calculated as 750 000 persons per year^[8]. As previously demonstrated by Correa *et al.*, atrophic gastritis and the following intestinal metaplasia are regarded as an essential status for intestinal type cancer development^[9]. It has been widely accepted that there is a strong association between *H pylori*-associated gastritis and gastric cancer. Nomura *et al.* and Parsonnet *et al.* first reported the relationship between *H pylori* infection and gastric cancer in 1991^[10,11]. In 1994, the International Center for Cancer Research officially recognized that *H pylori* was a definite carcinogen for gastric cancer on the basis of several epidemiological reports^[12]. Moreover, Huang *et al.* demonstrated the relationship between *H pylori* seropositivity and gastric cancer by meta-

analysis^[13]. The odds ratios for gastric cancer were calculated as 1.92 (1.32-2.78; 95%CI), 2.24 (1.15-4.4), and 1.81 (1.16-2.84) for all studies, cohort and case-control studies, respectively. In addition, many investigations showed the tight relationship between *H pylori* and not only intestinal type cancer, but also diffuse type cancer^[13,14]. Even in gastric cancer at a younger age, we were able to detect a tight relationship between gastric cancer and *H pylori*^[15]. In 2001, Uemura *et al.*^[16] clearly demonstrated that gastric cancer developed only in patients with *H pylori* infection with a prospective study. They prospectively followed 1246 Japanese people for 7.8 years and found that gastric cancer had been detected in only 36 patients with *H pylori* infection. No gastric cancer was found in *H pylori*-negative patients in their study.

These findings strongly suggest the implication of *H pylori* in gastric carcinogenesis. Next, we should clarify the hypothesis as to whether we can control gastric carcinogenesis by the eradication of *H pylori*. In the present study, we have summarized the recent clinical evidence in this field and have attempted to answer this question.

Gastric inflammation induced by *Helicobacter pylori* infection and its carcinogenic effects

Persistent infection of *H pylori* induces the characteristic inflammation in the gastric mucosa with the histological finding of mononuclear cell/neutrophil infiltration. In particular, the superoxides, such as nitric oxide, in the gastric mucosa play an important role in the initiation of the carcinogenesis as a mediator of carcinogenic nitrosamine formation, DNA damage and tissue injury. Studies have also revealed that *H pylori* infection in human beings is associated with the enhanced expression of iNOS by tissue neutrophils and mononuclear cells^[17,18]. Previously, we have reported that the expression of inducible nitric oxide synthase and nitrotyrosine in chronic gastritis is a predictive marker for a high risk of gastric cancer development^[19]. The iNOS-producing gastritis, which is supposed to be strongly associated with gastric cancer, showed a characteristic cytokine profile and serum gastrin pattern^[20].

Chronic inflammation results in the destruction of parietal cells of the oxyntic gland in the gastric corpus followed by the alteration of the pathophysiological status, including gastric acid secretion^[21]. The pH of gastric juice is a major determinant of the nitrite and vitamin C concentrations in gastric juice. Previous studies reported a close linkage between low acid output and an increased concentration of nitrite and N-nitroso compounds in gastric juice^[22]. Vitamin C was actively secreted into gastric juice and scavenges nitrite from the gastric juice and thereby acts to prevent the formation of nitrosamines. Vitamin C concentrations in gastric juice were found to be low in patients with hypochlorhydria status^[23]. In addition, we have reported that gastric juice nitrite concentrations are higher and vitamin C concentrations are lower in patients with gastric cancer than those in atrophic gastritis, despite similar intra-gastric pH and *H pylori* status^[24].

Furthermore, inflammation-derived cytokines and growth factors are known to act as promotion/proliferation factors for gastric cancer cells. Especially,

hepatocyte growth factor has been reported to play a crucial role in epithelial/tumor cell proliferation, and its expression was promoted in *H pylori*-infected mucosa^[25]. Churin *et al.* reported that *H pylori* activated c-Met (the receptor of HGF) in AGS cells, suggesting the important role of HGF in an autocrine manner^[26]. In addition, the regulation of apoptotic signals is another important factor for the promotion/proliferation of cancer cells. *H pylori* was reported to be able to induce apoptosis of the gastric epithelial cells directly^[27]. Nagasako *et al.*^[28] have demonstrated that the Smad-5, which contributes to the apoptosis of gastric epithelial cells, is upregulated by *H pylori* infection.

Many recent advances have revealed the detailed molecular mechanisms of gastric inflammation induced by *H pylori*. The research on some virulent bacterial factors, VacA and CagA, were reported successively from Japanese groups. Fujikawa *et al.*^[29] clarified that VacA binds to protein tyrosine phosphatase receptor type Z (Ptpz), which is a specific receptor on epithelial cells, and induces the intracellular pathway *via* G protein-coupled receptor kinase-interactor 1 (Git1) and pleiotrophin. On the other hand, the advantage of CagA is the most sensational in this field. Previously, Huang *et al.*^[30] demonstrated the strong association between anti-CagA seropositivity and gastric carcinogenesis, suggesting the importance of CagA for gastric carcinogenesis. CagA protein produced in the bacterial cell is translocated into the host cell by a type IV secretory system^[31], followed by translocation to the membrane^[32], tyrosine phosphorylation of the EPIYA motif by *src*-family kinase and the activation of SHP-2 phosphatase, which is the important second messenger from CagA (Figure 1)^[33]. Recent studies by Hatakeyama *et al.* clarified that the CagA protein showed diversity and was subclassified into two types, the Western type and East-Asian type, and the later type was reported to have a high affinity to SHP-2 and was regarded as a more harmful form^[34]. It is possible to explain the international diversity of the prevalence of gastric cancer; in Western countries, the prevalence of gastric cancer is relatively low because Western type CagA (or Cag negative strain) is dominant. On the other hand in East-Asian countries, the more virulent strain (East-Asian type) is the major strain, and this is a reason for the higher prevalence of gastric cancer. However, it is still unclear why some Japanese/Chinese people do not show corpus atrophy, even if they carry the East-Asian type CagA. Mimuro *et al.*^[35] suggested another signal pathway via growth factor receptor bound 2(Grb2), which is independent of CagA phosphorylation. Intra-gastric diversity or other bacterial factors must be examined to solve this question, as well as the host factors and environmental factors including a high intake of salt^[36].

After successful *H pylori* eradication therapy, these harmful conditions are dramatically improved. There is no doubt that the eradication therapy can diminish the risk of the new development of gastric cancer or progression from the pre-malignant status. However, it should be emphasized that it takes a long time from when one cell transforms into a cancer cell to when we can detect the cancer tissue by endoscopic examination. Although the growth rate of gastric cancer cells differs

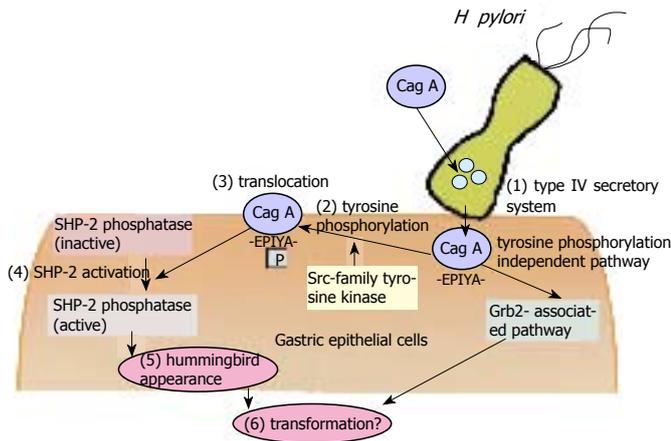


Figure 1 Intracellular molecular pathway starting from *H. pylori* infection.

according to their biological or histological characteristics, Haruma *et al.*^[37] reported that average doubling time of early gastric cancer is 16.6 mo in the polypoid type. From this fact, it is likely that when we detect the cancer lesion in the stomach by endoscopic examination, cancer cells must have already been present for many years, even if it is not detectable. Cancer cells have progressed through the multi-step process of genetic and epigenetic alteration of oncogenes^[38]. These alterations must occur in the cancer cells in the initial stage, and it is unlikely that these could be always cancelled by eradication therapy alone.

Reversibility of pre-malignant status; atrophic gastritis and intestinal metaplasia

Many reports have mentioned the improvement of neutrophil/lymphocyte infiltration after *H. pylori* eradication therapy. However, it is still controversial as to the improvement of glandular atrophy or intestinal metaplasia after the eradication therapy. Annibale *et al.* have concluded that eradication therapy does not improve mucosal atrophy^[39]. Sung *et al.*^[40] reported the results of a large-scale prospective randomized study and concluded that eradication therapy prevents the progression of atrophy, which was not reversible. However, some problems remain that the following period is relatively short in the former, and the basal grades of atrophy were mild in the latter study. On the other hand, Ohkusa *et al.*^[41] and Haruma *et al.*^[42] enrolled patients with atrophic gastritis and reported the reversibility of atrophy after *H. pylori* eradication therapy. Furthermore, we previously followed up 22 patients in whom *H. pylori* was eradicated for 5 years and confirmed that glandular atrophy is reversible both in the gastric corpus and in the antrum^[43]. It should be noted that the reversibility of atrophy was found in the patients with moderate atrophy, and it took a long-time to confirm the reversibility. In cases with complete disappearance of the gland, such as cases with gastric adenoma, reconstruction of the gland may be unlikely. Recently, Sugiyama *et al.*^[44] reported the regression of corpus atrophy after eradication. They emphasized the importance of the biopsy site, and demonstrated that the most suitable point is the lesser curvature of the gastric corpus.

Another major problem is the reversibility of the

intestinal metaplasia. Although some reports have refuted the improvement of intestinal metaplasia^[39,45], there has been a report suggesting the effect of eradication therapy in the improvement of intestinal metaplasia^[41]. Correa *et al.*^[46] reported that eradication therapy could regress not only the degree of atrophy, but also the intestinal metaplasia in a randomized controlled trial. Leung *et al.*^[47] demonstrated that the eradication therapy could prevent the progression of intestinal metaplasia using a randomized controlled trial with a 5-year follow-up. However, to evaluate the status of intestinal metaplasia, biopsy specimens for point-diagnosis seem to be not suitable. Therefore, we performed dye-endoscopy using methylene blue solution, which allowed us to evaluate the degree of intestinal metaplasia as a "field". Our overall results suggested the reversibility of intestinal metaplasia by following up over long periods, even if all patients did not show a regression of the intestinal metaplasia^[43]. At present, a large-scale prospective study (Japanese intervention trial of *H. pylori*) has been ongoing. In this study, the reversibility of atrophic gastritis was examined by endoscopic and histological examinations, and its final result will be published in 2006. Although the improvement of atrophic gastritis or intestinal metaplasia links to the regression of gastric carcinogenesis at the extremely early stage, a long-term (more than 10 years) follow-up study will be necessary to confirm the effect of eradication therapy on gastric carcinogenesis.

Does eradication therapy truly diminish gastric cancer?

It is clinically important to clarify whether gastric carcinogenesis could be influenced by eradication therapy. Firstly, Uemura *et al.*^[48] reported the reduced incidence of secondary developed gastric cancer by eradication therapy in patients with endoscopic resection of the primary gastric cancer. Because of the reason described above, their data did not suggest an anticarcinogenic effect of the eradication therapy, but instead an antiproliferative effect of cancer cell growth. Indeed, we have previously reported that the Ki-67 labeling index in cancer cells is lower in cancer lesions without *H. pylori* infection than in those with *H. pylori*^[49]. Recently, Take *et al.*^[50] reported that they followed up 1120 peptic ulcer patients with eradication therapy prospectively (mean 3.4 years) and found that gastric cancer is more frequently detected in patients with failed eradication than those with successful eradication. They have also demonstrated that gastric cancer was never detected in duodenal ulcer patients, in whom atrophic gastritis should be absent. On the other hand, a Chinese group has demonstrated, with a randomized controlled trial, that there is a relative decrease in cancer incidence in patients with eradication therapy in the overall population, but this difference did not reach a level of significance^[51]. Only in a subgroup without pre-cancerous lesions (without atrophy or intestinal metaplasia) they demonstrated the statistically reduced incidence of gastric cancer by eradication therapy. These results may be partially conflicting to each other and the reason for this is uncertain. However, we should pay attention to the difference in the clinical stage of gastric cancer detected and in the diagnostic ability of endoscopic examination

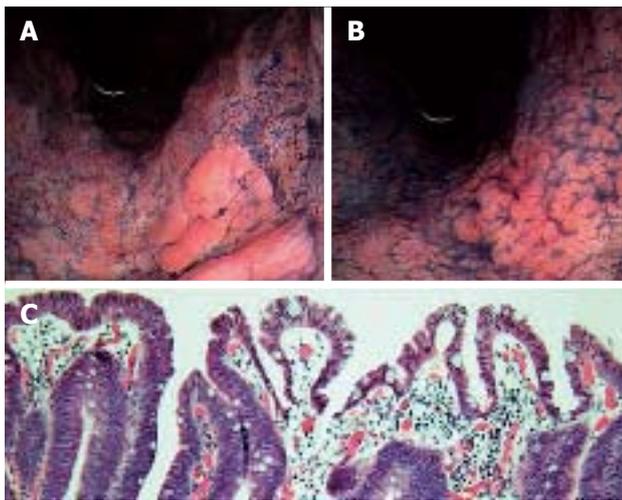


Figure 2 Dye-endoscopic features of gastric adenocarcinoma at pre- (A) and post-eradication therapy (B). Tumors became flattened and indistinct after eradication therapy. Histological features of gastric neoplasm at post-eradication are demonstrated in panel (C). Patient was a 67-year-old male.

between both trials. Whereas, in the Japanese study, most gastric cancer lesions were detected in the early stage as an intramucosal cancer, lesions found in Chinese study were those in a more advanced stage. We could not conclude that these two studies were designed with the same method because the endpoints may be different.

Recently, Kamada *et al.*^[52] also prospectively followed up 1 787 patients with eradication therapy (median 4.5 years) and demonstrated that gastric cancer could be detected in 20 patients (1.1%). Most cancer lesions were detected in the early stage as intramucosal cancer and its histology was intestinal type dominant (75%). We also prospectively examined 101 patients with atrophic gastritis prospectively for more than 60 mo (mean 63.2 months) and found gastric cancer incidence in 8 patients^[53]. Most gastric cancers were detected in the early stage and their histologies were of the intestinal type, and this is in complete agreement with previous studies. In addition, gastric cancer is more frequently found in elderly patients than in younger patients ($P < 0.05$). For the patients with atrophic gastritis, the age at the time of eradication therapy is an important factor for the occurrence of cancer after successful eradication. The long-term strict follow-ups after eradication seem to be necessary in elderly patients with atrophic gastritis.

Does alteration of the tumor appearance after eradication therapy modulate the incidence of cancer discovery?

As described above, the focus must be placed on the diagnostic ability of gastric cancer by endoscopic examination when we discuss the cancer discovery rate determined clinically. One of the difficulties of this field seems to be based on the methodology used to evaluate gastric carcinogenesis. Researchers can evaluate the degree of carcinogenesis only by the discovery rate of gastric cancer by endoscopic examination. Due emphasis must be placed on the differences in the diagnostic ability of each examination.

Moreover, endoscopic morphology might be influenced

directly by eradication therapy, thus affecting the discovery rate of gastric cancer. Previously, Gotoda *et al.*^[51] reported the endoscopic regression of gastric adenoma after successful eradication therapy. If the eradication itself influences the tumor morphology, this may affect the tumor discovery rate. Then, we investigated the morphological changes in the gastric neoplasm after *H. pylori* eradication in Japanese patients. After a one-month follow-up, endoscopic re-evaluation revealed that one-third of the gastric tumor became indistinct and some tumors were difficult to discern with ordinary endoscopic observation^[55]. All these altered lesions were of the superficial-elevated type, which is the characteristic appearance of intestinal type cancer, irrespective of the grade of histological appearance (adenoma or carcinoma) (Figure 2). The tumor appearance became flattened and the height of the lesions decreased, then the tumor became indistinct after eradication, even after a short time. In the depressed type cancer, that is typical for diffuse type gastric cancer, we could not find any morphological changes after eradication^[55]. These results suggest that the morphology of the gastric neoplasm changes after eradication in the short-term, especially in the intestinal type gastric cancer, which were found characteristically in patients after eradication therapy. Even if the true incidence of cancer is not affected by eradication, the incidence of cancer discovery would be influenced by successful eradication therapy in cases of intestinal type cancer with elevated tumor features. Furthermore, we detected normal columnar epithelium over the neoplasm in some lesions (Figure 2). The appearance of normal foveolar epithelium must make it more difficult to detect gastric cancer by endoscopic observation. We could not explain the origin of this strange histological feature, but the most probable origin may be a regenerative change against the surface injury of the mucosal tumor tissue induced by the improved acid output after successful eradication. This must also contribute to the reduction in the rate of cancer discovery after successful eradication therapy and these overall alterations are quite rational for the explanation of the results reported by the Japanese researchers.

The mechanism of the antitumor promoting effect of *H. pylori* eradication therapy is still unknown. In the *in vitro* studies, *H. pylori* itself has been found to modify the expressions of several genes in gastric carcinoma cells^[56]. Semino-Mora *et al.*^[57] demonstrated the presence of *H. pylori*-derived toxic proteins and mRNAs in gastric tumor cells *in vivo*. However, their theory is still controversial, and, until now, it has been believed that *H. pylori* cannot exist in the gastric carcinoma cells. Thus, it is likely that *H. pylori* indirectly influences tumor cell growth by regulating the inflammatory reaction around the tumor tissue. Several cytokines have been induced by *H. pylori* infection and some of them may act as growth factors for tumor cells^[25,58]. Suzuki *et al.*^[59] reported the decreased level of HGF in the gastric mucosa after eradication, which was linked to the decreased cell turnover. These indicate the importance of gastric inflammation in the gastric mucosa rather than *H. pylori* itself on the luminal side. Gastrin is known to be an important gut-related hormone and a

growth factor for gastric cancer cells, and gastric tumor cells have been shown to contain its receptor^[60]. However, it is unlikely that our new findings of the morphological changes were induced by a gastrin-related system^[55].

There are two ways in which gastric tumors may grow; one is invasive downward growth and the other is expansive growth in the upward (luminal) direction. The latter may include the reactive (non-neoplastic) factor, which may be regulated by the gastric inflammation induced by *H pylori* infection. Kamada *et al.*^[52] analyzed the macroscopic features of gastric cancers discovered after eradication therapy and demonstrated that most lesions are of the depressed type. If the eradication therapy mainly influences the expansive growth, the true biological behavior of the gastric malignancies may not be improved by eradication therapy.

Clinical manifestations of *H pylori* eradication therapy and what are the problems in the next step?

There is no doubt that eradication therapy diminishes the prevalence of cancer development. In the animal model with Mongolian gerbils, Shimizu *et al.*^[61] demonstrated that *H pylori* eradication therapy diminishes the prevalence of gastric cancer incidence induced by *H pylori* infection and low-dose chemical carcinogen. Tatematsu *et al.*^[62] also reported that *H pylori* eradication therapy regresses the heterotopic proliferative glands in the gastric mucosa of Mongolian gerbils, suggesting that the eradication reduces the promoting effect of the bacterium.

In human studies, we cannot discuss the anticarcinogenic effect of eradication therapy unless we follow up patients for a sufficiently long period (more than 10 years). As discussed above, it is very important that we distinguish the “development” of gastric cancer from the “discovery” of the tumor. In addition, it is clinically important to clarify whether eradication therapy can diminish the mortality rate for gastric cancer or not. It is very critical to know as to how we regress the biologically malignant gastric cancer, including diffuse-type cancer in younger patients. No reports have discussed this, and further study is necessary to answer this question.

Another problem to be solved is the “point of no return” of gastric carcinogenesis by eradication therapy. In the animal model, earlier eradication was demonstrated to be more effective for preventing the development of gastric cancer^[63]. Since gastric cancer after eradication was frequently found in patients who received eradication in older age, we should consider that younger people should receive eradication therapy as early as possible. For the effective eradication for cancer prevention to be practical and economical, the selection of the population at higher risk should be clarified. It is especially necessary to identify the fundamental status of gastric inflammation in the development of diffuse type gastric cancer. Recent studies revealed that a high odds ratio for gastric cancer discovery was noticed in patients with nodular gastritis, which may be an important background for diffuse-type cancer^[64,65]. This type of gastritis should be an important target for earlier eradication therapy to reduce cancer death. In addition, we should determine useful biomarkers for gastric inflammation, which would be beneficial for real clinical practice^[66].

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Incidence and mortality of gastric cancer in China

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Abstract

Gastric cancer is one of the most frequent cancers in the world; almost two-thirds of gastric cancer cases and deaths occur in less developed regions. In China, based on two national mortality surveys conducted in 1970s and 1990s, there is an obvious clustering of geographical distribution of gastric cancer in the country, with the high mortality being mostly located in rural areas, especially in Gansu, Henan, Hebei, Shanxi and Shaanxi Provinces in the middle-western part of China. Despite a slight increase from the 1970s to early 1990s, remarkable declines in gastric cancer mortality were noticed in almost the entire population during the last decade in China. These declines were largely due to the dramatic improvements in the social-economic environment, lifestyle, nutrition, education and health care system after economic reforms started two decades ago. Nevertheless, gastric cancer will remain a significant cancer burden currently and be one of the key issues in cancer prevention and control strategy in China. It was predicted that, in 2005, 0.3 million deaths and 0.4 million new cases from gastric cancer would rank the third most common cancer. The essential package of the prevention and control strategy for gastric cancer in China would focus on controlling *Helicobacter pylori* (*H. pylori*) infection, improving educational levels, advocating healthy diet and anti-tobacco campaign, searching for cost-effective early detection, diagnosis and treatment programs including approaches for curable management and palliative care.

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Key words: Gastric cancer; Incidence; Mortality

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GASTRIC CANCER IN THE WORLD

Gastric cancer is one of the most frequent cancers in the world, in both men and women. In the year 2002, the age standardized incidence rate was 22.0 per 100 000 in men, and 10.4 per 100 000 in women, and mortality rate was 16.3 per 100 000 in men, and 7.9 per 100 000 in women, according to the latest global estimation-GLOBOCAN 2002^[1]. Despite a marked decrease, especially in mortality rate in many countries, the absolute number of gastric cancer cases and deaths is still a big burden of the local health program, since the world population and life expectancy are increasing. In 2002, it was estimated that there were 0.9 million new gastric cancer cases (0.60 million in men and 0.33 million in women) and 0.7 million deaths (0.45 million in men and 0.25 in women) from gastric cancer in the world. In men, gastric cancer ranks the third among the commonest cancers in incidence rate (after cancers of lung and prostate) and the second in mortality rate (after lung cancer), while in women, it is the fifth most common cancer in incidence and the fourth in mortality (after cancers of breast, cervix, lung and/or colon-rectum)^[1].

In terms of geographic distribution, almost two-thirds of gastric cancer cases and deaths occur in less developed regions. High rates apply to Japan, China, Korea, Central and South America, Eastern Europe, and parts of the Middle East, and low rates to North America, Australia and New Zealand, Northern Europe, and India^[1,2]. Five-year relative survivals of around 20% or less are frequently reported. The incidence ratios of men to women generally range between 1.5 and 2.5, with higher ratios for intestinal than diffuse cancers and in high-risk populations^[2].

NATIONAL GASTRIC CANCER MORTALITY PATTERN IN THE 1970s AND 1990s

In China, two national mortality surveys were conducted in 1973-1975 and 1990-1992, respectively, both organized by the National Office for Cancer Prevention of Control, the Ministry of Public Health, China. These two surveys showed the detail pattern and distribution of mortality rates for cancers in China at that time^[3-7]. The first mortality survey was a nation-wide study. An atlas was published based on the survey results^[4], providing a visual geographic distribution of gastric cancer profile in early 1970s. A map of the gastric cancer mortality distribution for males is shown in (Figure 1) as an example. An obvious clustering of geographical distribution of gastric cancer appears in China, with the high mortality

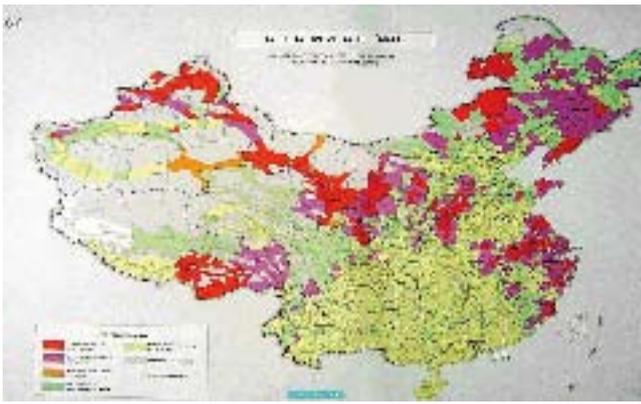


Figure 1 Mortality rates of gastric cancer in men in China, 1973-1975^[4].

Table 2 Changes in the age-standardized mortality rates of gastric cancer between 1973 and 1975 and between 1990 and 1992 in China (rate per 100 000)

| Area | Year 1973-1975 | | | Year 1990-1992 | | | Changes (%) | | |
|---------------|----------------|-------|-------|----------------|-------|-------|-------------|-------|-------|
| | Men | Women | Total | Men | Women | Total | Men | Women | Total |
| Whole country | 27.1 | 13.0 | 19.8 | 30.1 | 13.8 | 21.8 | 11.0 | 6.3 | 10.0 |
| Urban | 27.3 | 13.3 | 20.1 | 21.2 | 9.8 | 15.3 | -22.2 | -26.7 | -23.8 |
| Rural | 26.7 | 12.6 | 19.4 | 33.7 | 15.4 | 24.4 | 26.4 | 22.1 | 25.8 |

being mostly located in the north (Liaodong Peninsula, Shandong Peninsula, Yangtze River Delta and middle-western provinces along Taihang Mountain and 'Hexi Zoulang').

The second mortality survey covered 10% of the whole population that proved as a representative sample to reflect national cancer profile^[5-7]. The crude mortality rate of gastric cancer in China was 25.2 per 100 000 (32.8 per 100 000 in men and 17.0 per 100 000 in women), which accounted for 23.2% of the total cancer deaths in 1990-1992, making gastric cancer the first leading cause of cancer death^[8]. The geographic variety in the distribution was similar to that in the first survey in the 1970s. Table 1 shows the first 20 areas with high mortality rates for the gastric cancer (rates adjusted by the 1982 national census population). Most of these high-risk areas are located in rural areas, especially in Gansu, Henan, Hebei, Shanxi and Shaanxi Provinces in the middle-western part of China.

By comparing the above two national mortality surveys, an increasing trend for the age-standardized mortality rates of gastric cancer was shown in the entire population in China during the two decades. Nevertheless, the increase only appeared in rural areas, while a decreasing trend was noticed in urban population^[9,10] (Table 2). In terms of age-specific mortality rates, a decreasing trend was noticed among almost all age groups (except age group 70-79 years) in urban residents, while an increasing trend was seen among most of age groups (except age group 30-44 years) in rural areas^[10]. Nevertheless, with almost two-thirds of the population reside in rural areas, gastric cancer was still the first most common cancer in the entire population in China during 1970s and early 1990s,

Table 1 The first 20 areas with high mortality rate for gastric cancer in China - according to the second national mortality survey in 1990-1992 (rate per 100 000)

| Area | Rate | Area | Rate |
|--------------------------|--------|-------------------------------------|-------|
| Wuwei town, Gansu | 117.78 | Jiyuan town, Henan | 66.59 |
| Yangcheng county, Shanxi | 104.01 | Zhangye town, Gansu | 64.43 |
| Pingshun county, Shanxi | 96.95 | Jiaxian county, Shaanxi | 64.36 |
| Changle county, Fujian | 93.38 | Tianchang county, Anhui | 63.04 |
| Shexian county, Hebei | 85.64 | Tianzhu Tibet Autho county, Gansu | 59.18 |
| Lujiang county, Anhui | 84.46 | Putian county, Fujian | 58.83 |
| Zanghuang county, Hebei | 77.67 | Linxian county, Henan | 58.56 |
| Neixiang county, Henan | 77.45 | Wudu county, Gansu | 58.54 |
| Yuanqu county, Shanxi | 74.22 | Tianjian Dist., Fuzhou city, Fujian | 57.52 |
| Linze county, Gansu | 66.69 | Xianju county, Zhejiang | 53.99 |

although it dropped to the third in urban areas.

In addition to the above two national mortality surveys, there were a few publications on gastric cancer mortality trends at local levels, either in big cities such as Beijing, Shanghai, or in high-risk areas such as in Henan and Hebei Provinces^[11-14]. Similarly, the mortality rates of gastric cancer remain high although there was a slight decreasing trend during different time periods among those areas. Therefore, gastric cancer is still the major common cancer and a big burden for local health resources and facilities.

GASTRIC CANCER INCIDENCE AND MORTALITY PATTERNS AT THE NATIONAL LEVEL IN RECENT YEARS

Based on a routine mortality reporting system, covering 10% of the Chinese population, from the Ministry of Public Health which was submitted to the World Health Organization^[15], the gastric cancer mortality trends in China were analyzed from 1987 to 1999, using the joinpoint model. The trends were further combined with the ratio of incidence to mortality from data in seven cancer registries in China that were published in the 8th edition of Cancer Incidence in Five Continents^[16], to estimate and project the mortality and incidence for gastric cancer in 2000 and 2005 (by site, age, sex and area) at the national level, using the log-linear regression model with Poisson distribution assumption. These results have been serially published recently^[17-19].

According to the data from the Center of Health Information Statistics (CHIS) under the Ministry of Public Health, gastric cancer mortality rates were higher in rural than urban areas, and in men than in women. During 1987-1999, slight but significant declines in mortality were noticed in almost the entire subpopulation (except in urban men before 1991) and for most age groups (except age group 15-34) in rural areas (Figures 2 and 3). In 2000, 0.3 million deaths and 0.4 million new cases were estimated for gastric cancer, similar to what was projected in 2005, while the latter had a slight decrease in men but increase in women (Table 3). In 2005, in terms of incidence rates, gastric cancer would rank the third among the most common cancers in China (after cancers of lung

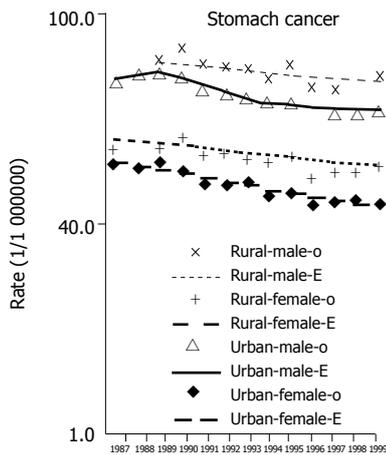


Figure 2 Time trends for age-standardized mortality rates (both observed and expected by model) of gastric cancer during 1987-1999 in China, by areas (rural and urban) and sex (per 100 000)

Table 3 Gastric cancer incidence and mortality in China in 2000 and 2005

| | Men | | Women | |
|-------------------------|-----------|-----------|-----------|-----------|
| | Year 2000 | Year 2005 | Year 2000 | Year 2005 |
| Mortality (per 100 000) | | | | |
| rates | | | | |
| Age 45-54 | 45.5 | 43.3 | 20.5 | 19.0 |
| Age 55-64 | 109.4 | 89.4 | 46.6 | 40.1 |
| Age 65-74 | 229.4 | 198.3 | 104.6 | 91.3 |
| Age 75+ | 289.6 | 261.9 | 133.3 | 116.3 |
| ASR ¹ | 32.7 | 28.8 | 15.0 | 13.3 |
| Number of deaths | 200 518 | 197 222 | 95 942 | 97 843 |
| Incidence (per 100 000) | | | | |
| rates | | | | |
| Age 45-54 | 70 | 66.7 | 31.6 | 29.3 |
| Age 55-64 | 145.7 | 119 | 62.1 | 53.4 |
| Age 65-74 | 264.3 | 228.5 | 120.5 | 105.2 |
| Age 75+ | 288.8 | 261.2 | 133 | 116 |
| ASR ¹ | 41.9 | 37.1 | 19.5 | 17.4 |
| Number of cases | 256 256 | 253 110 | 121 485 | 123 883 |

¹ASR: Age-standardized rate (adjusted by the world standard population).

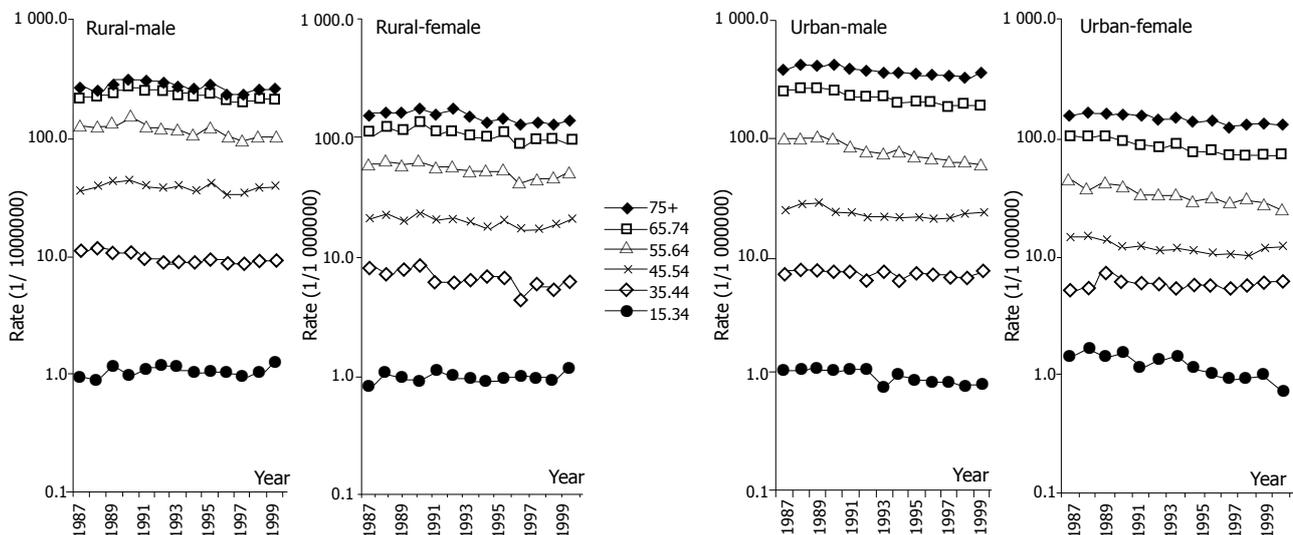


Figure 3 Time trends for age-specific mortality rates of gastric cancer during 1987-1999 in China, by areas (rural and urban) and sex, in CHIS data (rates per 100 000).

and liver in men and after cancers of breast and lung in women)^[19].

FACTORS INFLUENCING THE GASTRIC CANCER PATTERN IN CHINA

Despite a slight increase from 1970s to early 1990s, remarkable declines in gastric cancer mortality were shown during the last decade in China. These declines were largely due to the dramatic improvements in the social-economic environment, lifestyle, nutrition, education and health care system after economic reforms started two decades ago. These include better socio-economic circumstances, higher educational levels, better refrigeration, reduced consumption of salted, smoked, and chemically preserved foods, eating more fruit and vegetables and remarkably improved sanitary conditions of the house and living standards, supplement intake nutrients such as vitamin C, vitamin E, beta-carotene, selenium and decreased intake of nitrosamine, which was strongly suspected as a major

risk factor for gastric cancer^[20-31]. *H pylori* infection, which is defined by the World Health Organization as a definite gastric carcinogen, is linked to crowded living conditions, family size, sharing a bedroom, low socio-economic status, low educational level and poor sanitation, and infrequent hand washing before meals. The prevalence of *H pylori* infection has reduced dramatically due to the improved socio-economic status and lifestyle changes during last decades, especially among urban areas. In addition, widespread prescription of antibiotics may be responsible for reducing *H pylori* infection. Finally, some locally popular customs, such as drinking green tea, consuming tofu, and ginger have been suggested to have a possible protective effect on gastric cancer^[32-35].

However, huge demographic changes in China make the total number of incident cases and deaths from gastric cancer slightly declined in men between 2000 and 2005, while the number increased in women, despite the significant declining trends in rates among all age groups (Table 2)^[17-19].

CONCLUSION

It is estimated that gastric cancer currently ranks the third among most common cancers, and will remain a significant cancer burden in China during the next decade. It will be, undoubtedly, one of the keys in cancer prevention and control strategy in China. The essential package would focus on controlling *H pylori* infection, improving educational levels, advocating healthy diet and anti-tobacco campaign, searching for cost-effective early detection, diagnosis and treatment programs including approaches for curable management and palliative care^[56].

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Berberine induces cell cycle arrest and apoptosis in human gastric carcinoma SNU-5 cell line

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of Bcl-2, release of Ca²⁺, decreased the mitochondrial membrane potential and then led to the release of mitochondrial cytochrome C into the cytoplasm and caused the activation of caspase-3, and finally led to the occurrence of apoptosis.

CONCLUSION: Berberine induces p53 expression and leads to the decrease of the mitochondrial membrane potential, Cytochrome C release and activation of caspase-3 for the induction of apoptosis.

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Key words: Berberine; Cell cycle; Apoptosis; Caspase-3; ROS; MMP; SNU-5 cells

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Abstract

AIM: To investigate the relationship between the inhibited growth (cytotoxic activity) of berberine and apoptotic pathway with its molecular mechanism of action.

METHODS: The *in vitro* cytotoxic techniques were complemented by cell cycle analysis and determination of sub-G₁ for apoptosis in human gastric carcinoma SNU-5 cells. Percentage of viable cells, cell cycle, and sub-G₁ group (apoptosis) were examined and determined by the flow cytometric methods. The associated proteins for cell cycle arrest and apoptosis were examined by Western blotting.

RESULTS: For SNU-5 cell line, the IC (50) was found to be 48 μmol/L of berberine. In SNU-5 cells treated with 25-200 μmol/L berberine, G₂/M cell cycle arrest was observed which was associated with a marked increment of the expression of p53, Wee1 and CDk1 proteins and decreased cyclin B. A concentration-dependent decrease of cells in G₀/G₁ phase and an increase in G₂/M phase were detected. In addition, apoptosis detected as sub-G₀ cell population in cell cycle measurement was proved in 25-200 μmol/L berberine-treated cells by monitoring the apoptotic pathway. Apoptosis was identified by sub-G₀ cell population, and upregulation of Bax, downregulation

INTRODUCTION

The growth of tumor cells not like normal cells is uncontrolled. It is a strategy to change biological properties of cancer cells that lead to apoptosis of killing cancer cells in order to reach chemotherapeutic function for anticancer drugs. Apoptosis is a physiological mode of cell death, which can be selectively triggered by cells in response to the stimuli^[1]. Therefore, the induction of apoptosis is a key factor for anticancer drugs.

Berberine (5,6-dihydro-9,10-dimethoxybenzo[g]-1,3-benzodioxole[5,6-a]quinolizinium), a kind of alkaloid, was initially isolated from a Chinese herbal medicine and used as an antibiotic long ago; and it has effects against many bacterial species^[2,3]. In the past years, berberine has subsequently been examined for anticancer activity following evidence of antineoplastic properties^[3-5]. It has also been shown that berberine interacts with nucleic acids especially DNA^[6] *in vitro*. Berberine exhibits the ability to induce apoptosis in human cancer cells^[5,7] and promyelocytic leukemia HL-60 cells can form berberine complexes with DNA^[8].

Cell cycle studies showed that berberine induces rapid apoptosis in a subpopulation (S phase) of the cells^[8]. It is

also reported that berberine has dose-dependent effects of berberine on G₂/M phase and apoptosis in Balb/c 3T3 cells^[9]. However, the effects of berberine on human gastric cells are still unclear. Therefore, the purpose of the present study was to find out the molecular mechanism of berberine underlying human gastric cancer cell line (SNU-5).

MATERIALS AND METHODS

Materials

Berberine, propidium iodide (PI), Tris-HCl, trypan blue, ribonuclease-A and Triton X-100 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Potassium phosphates, dimethyl sulfoxide (DMSO), and TE buffer were purchased from Merck Co. (Darmstadt, Germany). Iscove's modified Dulbecco's medium, glutamine, fetal bovine serum (FBS), and penicillin-streptomycin, trypsin-EDTA were obtained from Gibco BRL (Grand Island, NY, USA).

Human gastric carcinoma cell line (SNU-5)

SNU-5 cell line (human gastric carcinoma; 33 years, female) was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were immediately placed into 75 cm × 75 cm × 75 cm tissue culture flasks and grown at 37 °C under a humidified 50 mL/L CO₂ and 950 mL/L air in 800 mL/L Iscove's modified Dulbecco's medium supplemented with 200 mL/L FBS, 10 g/L penicillin-streptomycin (1 MU/L penicillin and 10 g/L streptomycin) and 10 g/L glutamine as described previously^[10].

Measurement of cell viability after cells were co-treated with berberine determined by trypan blue exclusion and flow cytometry

SNU-5 cells were plated in 12-well plates at a density of 5 × 10⁵ cells/well and grown for 24 h. Various concentrations of berberine were added to the cells for final concentrations of 0, 25, 50, 100, and 200 μmol/L, while only DMSO (solvent) was added for the control regivnen and grown for a different period of time at 37 °C, was added 50 mL/L CO₂ and 950 mL/L. The trypan blue exclusion and flow cytometry protocols were used as previously described for determining cell viability^[7].

Flow cytometry analysis of DNA content for cell cycle and apoptosis analysis in SNU-5 cells co-treated with different concentrations of berberine

About 5 × 10⁵ SNU-5 cells/well in 12-well plates were incubated with berberine (0, 25, 50, 100, and 200 μmol/L) for different time periods before the cells were harvested by centrifugation. The cells were fixed gently (drop by drop) in 700 mL/L ethanol (in PBS) in ice overnight at -20 °C and then re-suspended in PBS containing 40 g/L PI, 0.1 g/L RNase (Sigma) and 0/10 g/L Triton X-100. After 30 min at 37 °C in the dark, the cells were transferred to the tube, analyzed with flow cytometry (Becton-Dickinson, San Jose, CA, USA) equipped with an argon laser at 488 nm. Then cell cycle and apoptosis were determined and analyzed^[7,10].

Inhibition of berberine-induced apoptosis by caspase inhibitor z-VAD-fmk in SNU-5 cells

In order to further examine whether caspase-3 activation was involved in apoptosis triggered by berberine, SNU-5 cells were pretreated with the cell permeable broad-spectrum caspase inhibitor z-VAD-fmk 3 h prior to the treatment with 100 μmol/L berberine. Apoptosis and caspase-3 activity were then determined as described above.

Detection of reactive oxygen species (ROS) in SNU-5 cells co-treated with berberine by flow cytometry

The level of ROS in the SNU-5 cells was examined and quantitated by flow cytometry (Becton Dickinson FACS Calibur), using 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma). SNU-5 cells were treated with or without berberine (100 mmol/L) for 0, 0.5, 1, 1.5, 2, 4, 6, and 12 h to detect the changes of ROS. The cells were harvested and washed twice, re-suspended in 500 mL of 2,7-dichlorodihydrofluorescein diacetate (10 μmol/L) and incubated at 37 °C for 30 min and analyzed by flow cytometry as described previously^[11].

Detection of Ca²⁺ concentrations in SNU-5 cells co-treated with berberine by flow cytometry

The level of Ca²⁺ in the SNU-5 cells was determined and quantitated by flow cytometry (Becton Dickinson FACS Calibur), using the Indo 1/AM (Calbiochem; La Jolla, CA, USA). Cells were treated with or without berberine (100 mmol/L) for 0, 0.5, 1, 1.5, 2, 4, 6, and 12 h to detect the changes of Ca²⁺ concentrations. The cells were harvested and washed twice, and re-suspended in Indo 1/AM (3 mg/L) and incubated at 37 °C for 30 min and analyzed by flow cytometry as described previously^[12].

Detection of mitochondrial membrane potential in SNU-5 cells co-treated with berberine by flow cytometry

The level of mitochondrial membrane potential in the SNU-5 cells was determined by flow cytometry (Becton Dickinson FACS Calibur), using DiOC₆ (4 mol/L) (Calbiochem, Inc., La Jolla, CA, USA). Cells were treated with or without various concentrations (0, 25, 50, 100, and 200 mmol/L) of berberine for 1, 2, 4, 6, 12, 24 h to detect the changes of mitochondrial membrane potential. The cells were harvested and washed twice, re-suspended in 500 mL of DiOC₆ (4 mol/L) and incubated at 37 °C for 30 min and analyzed by flow cytometry^[11].

Detection of caspase-3 activity and apoptosis in SNU-5 cells co-treated with berberine by flow cytometry

The caspase-3 activity and apoptosis in the SNU-5 cells were determined by flow cytometry (Becton Dickinson FACS Calibur), using PhiPhiLux-G₂D₂ (4 × 10⁻⁴ mmol/L) (OncoImmunin, Inc., MD, USA). Cells were treated with or without various concentrations (0, 25, 50, 100, and 200 mmol/L) of berberine and co-treated with or without caspase-3 inhibitor (z-VAD-fmk) for 24 h to detect the changes of caspase-3 activity and apoptosis. The cells were harvested and washed twice, re-suspended in 50 mL PhiPhiLux-G₂D₂ of (4 × 10⁻⁴ mmol/L) and incubated at

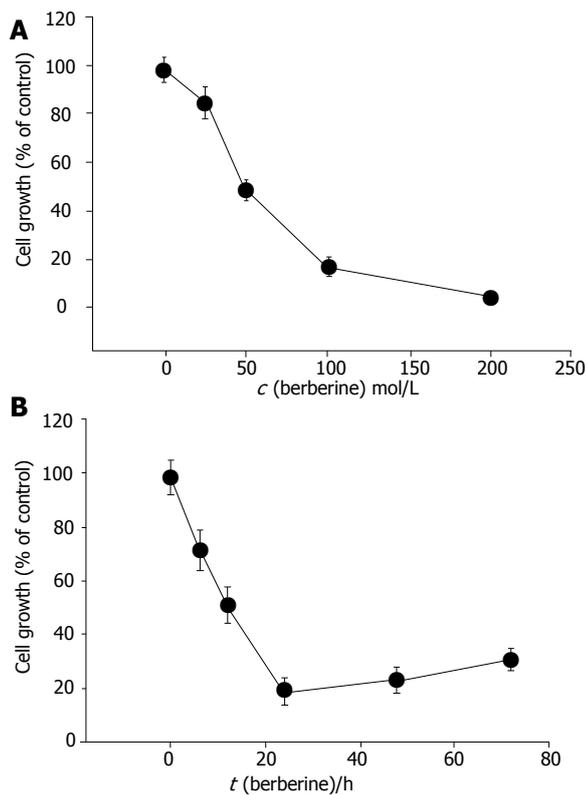


Figure 1 Percentage of viable SNU-5 cells treated with berberine with 24-h incubation. The SNU-5 cells (2×10^5 cells/well; 12-well plate) were plated in 80% Iscove's modified Dulbecco's medium+20% FBS with different concentrations of berberine for 24 h (Panel A) or 100 μ mol/L berberine for 6, 12, 24, 48, and 72 h (Panel B). Then the cells were collected by centrifugation and the viable cells were determined by trypan blue exclusion and flow cytometry as described in Materials and Methods. Each point is mean \pm SD of three experiments.

37 $^{\circ}$ C for 30 min and analyzed by flow cytometry as described previously^[11].

Effect of berberine on CDK1, Wee1, Cdc25, p53, JNK, Bcl-2, Bax, and cytochrome C of SNU-5 cells

The total protein was collected from SNU-5 cells treated with or without various concentrations of berberine for 48 h before CDK1, Wee1, Cdc25, p53, JNK, Bcl-2, Bax, and cytochrome C were measured by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot as described previously^[12,13].

Statistical analysis

Student's *t*-test was used in statistical analysis between berberine-treated and control groups. $P < 0.05$ was considered statistically significant

RESULTS

Effects of berberine on growth of SNU-5 cells

Percentage of cell growth was significantly different between the berberine-treated group and control group. The effects of berberine on SNU-5 cells were dose-dependent (Figure 1A). Increasing the time of incubation led to the decrease of percentage of cell growth (Figure 1B). Apparently the effects of berberine on SNU-5 cells also were time dependent.

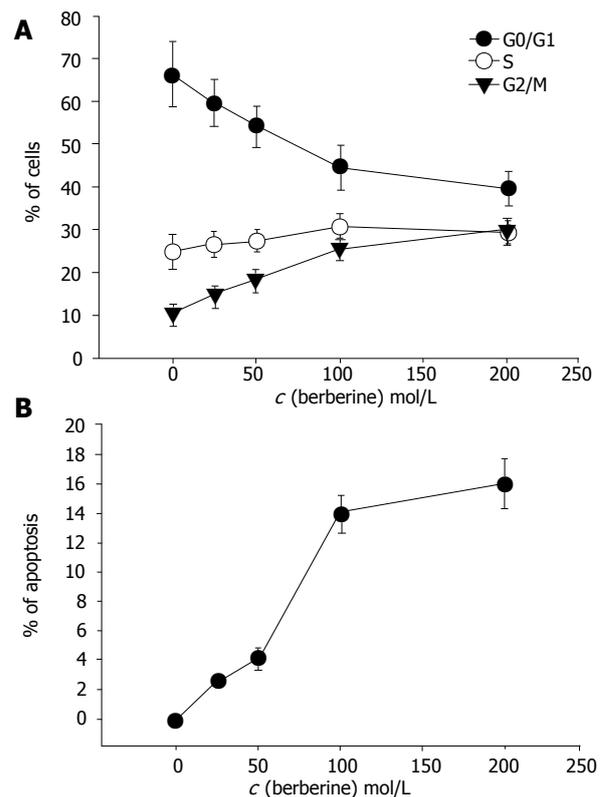


Figure 2 Flow cytometric analysis of the effects of berberine on SNU-5 cell cycle and sub-G₁ group. The SNU-5 cells were exposed to various concentrations of berberine for 48 h, and the cells were harvested and analyzed for cell cycle (Panel A: the percent of cells in phase) and sub-G₁ group (Panel B: the percent of cells in apoptosis) were analyzed by flow cytometry as described in Materials and Methods. Data represents mean \pm SD of three experiments.

Cell cycle arrest and apoptosis induced by berberine in SNU-5 cells

First, we studied the cell cycle and occurrence of apoptosis induced by berberine. Cell cycle and apoptosis were detected by PI staining and annexin V method after 48 h of continuous exposure to berberine before analyzed by flow cytometry (Figures 2A and 2B). As shown in Figure 2, berberine induced G₂/M arrest and apoptosis in a concentration- and time-dependent manner.

Effect of berberine on cyclin B, CDK1, Wee1, and CDC25C of SNU-5 cells

Berberine increased the expression of Wee1 and CDC25C (Figures 3A and 3B) but decreased the expression of cyclin B and CDK1 (Figures 3C and 3D) as detected by western blotting.

Effects of berberine on the production of reactive oxygen species (ROS) in SNU-5 cell line

Percentage of ROS was significantly different between the berberine-treated group and control group. The effects of berberine on taking up of DCFH-DA dye by SNU-5 cells were dose-dependent (Table 1) and time dependent (Figure 4A).

Effects of berberine on the production of Ca²⁺ in SNU-5 cells

Percentage of Ca²⁺ concentrations was significantly

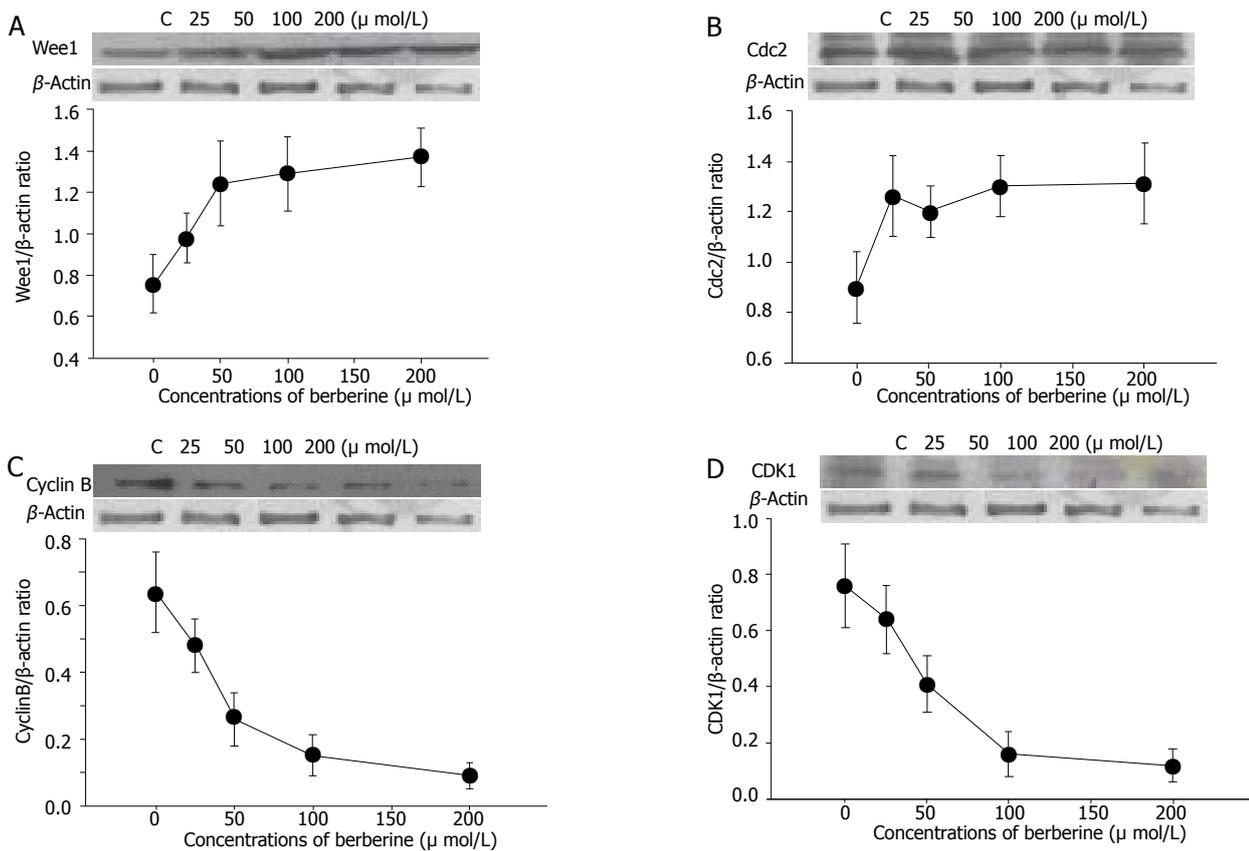


Figure 3 Changes of levels of CDK1 (A), cyclin B1 (B), Wee1 (C), and Cdc2 (D) in SNU-5 cells after exposure to berberine. SNU-5 cells ($5 \times 10^9 / L$) were treated with 0, 25, 50, 100, and, 200 $\mu\text{mol/L}$ berberine for 24 h, then cytosolic fraction and total protein were determined as described in Materials and Methods.

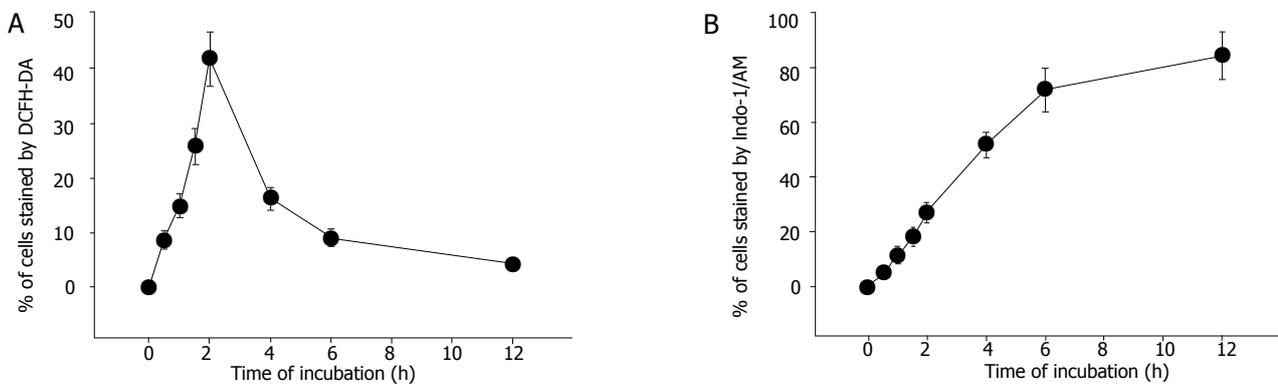


Figure 4 Flow cytometric analysis of reactive oxygen species (A) and Ca^{2+} concentration (B) in human gastric carcinoma SNU-5 cells with 100 $\mu\text{mol/L}$ berberine for various time periods. The SNU-5 cells (5×10^5 cells/mL) were treated with 100 $\mu\text{mol/L}$ berberine for 0, 0.5, 1, 1.5, 2, 4, 6, and 12 h to detect the changes of ROS and Ca^{2+} concentration. The zero concentration was defined as control. The percentage of cells stained with DCFH-DA dye was determined by flow cytometry as described in the Materials and Methods section.

different between the berberine-treated group and control group. The effects of berberine on taking up of Indo-1/AM dye by SNU-5 cells were dose and time-dependent (Table 1, Figure 4B).

Effects of berberine on the mitochondrial membrane potential in SNU-5 cells

Percentage of mitochondrial membrane potential (MMP) was significantly different between the berberine-treated group and control group. Apparently the effects of berberine on the levels of MMP determined by the take

up of the DiOL6 dye in SNU-5 cells were dose and time-dependent (Table 1, Figure 5).

Inhibition of berberine-induced caspase-3 activity and apoptosis by z-VAD-fmk in SNU-5 cells

The results indicate the caspase inhibitor that berberine increased caspase-3 activity in a dose- and time-dependent manner (Figure 6A). The SNU-5 cells were pretreated with the cell permeable broad-spectrum caspase inhibitor (z-VAD-fmk) 3 h prior to the treatment with berberine. The z-VAD-fmk decreased the caspase-3 activity. After

Table 1 Flow cytometric analysis of reactive oxygen species Ca^{2+} concentration and mitochondrial membrane potential in gastric carcinoma SNU-5 cells with treated various concentrations of berberine (mean \pm SD)

| Berberine (μ mol/L) | cells taking up DCFH-DA (%) | cells taking up Indo-1/AM (%) | cells taking up DiOC ₆ |
|--------------------------|-------------------------------|-------------------------------|-----------------------------------|
| 0 | 0.24 \pm 0.06 | 1.69 \pm 0.22 | 94.20 \pm 9.29 |
| 25 | 10.47 \pm 2.02 ^a | 28.78 \pm 2.49 ^a | 74.14 \pm 8.28 ^a |
| 50 | 28.18 \pm 3.14 ^a | 42.47 \pm 3.96 ^a | 40.21 \pm 5.08 ^a |
| 100 | 62.28 \pm 5.46 ^a | 74.62 \pm 6.48 ^a | 17.40 \pm 1.87 ^a |
| 200 | 79.49 \pm 7.08 ^a | 91.38 \pm 8.41 ^a | 8.16 \pm 1.04 ^a |

The SNU-5 cells (5×10^5 cells/mL) were treated with 0, 25, 50, 100, and 200 μ mol/L of berberine. The zero concentration was defined as control. The percentage of cells taking up DCFH-DA dye, was determined by flow cytometry as described in the Materials and Methods. ^a $P < 0.05$. *vs* control.

treated with berberine and z-VAD-fmk in SNU-5 cells, inhibition of berberine-mediated caspase-3 activation was accompanied with the marked attenuation of berberine-induced apoptotic cell death (Figure 6B).

Effect of berberine on p53, Bax, Bcl-2, and cytochrome C in SNU-5 cells

The results indicated that DADS increased the expression of p53 (Figure 3A), JNK phosphorylation (Figure 3B) and cytochrome C (Figure 3D) release but decreased the expression of Bcl-2 (Figure 3C) as detected by western blotting

DISCUSSION

Berberine (25–200 μ mol/L) was cytotoxic to SNU-5 cells in a dose- and time-dependent manner. The IC_{50} for SNU-5 cells was 48 μ mol/L (Figure 7). It is slightly different in HL-60 cells^[8]. But the sensitivity of murine leukemia L1210 cells to the berberine is higher than that of HeLa cells^[14]. Our results also showed that berberine induced ROS in a dose-dependent manner. It may be due to the cell death induced by ROS. Although it was demonstrated that berberine can decrease the intracellular ROS^[5], such variable effects are not uncommon. However, cells after treated with berberine for 48 and 72 h slightly increased their viability. They therefore may lead to the resistance to berberine due to the expression of multidrug-resistant transporters (mdr) because berberine can modulate expression of mdr1 gene product (pgp-170) that leads to reduced response to paclitaxel in digestive track cancer cells^[15].

Berberine arrests cells in S- and G₂/M-phase of the cell cycle, but the former effect is transient. However, G₂/M arrest is obvious. Apparently this effect is dose-dependent. Although it was reported that berberine could induce G₀/G₁ cell cycle arrest in murine L1210 cells^[14], it was also reported that berberine can induce G₂/M cell cycle arrest in Balb/c 3T3 cells^[4,16], suggesting that berberine induces cell cycle arrest depending on cell types. Therefore, the mechanism of berberine is not the same in all cell types. Western blot results from the present studies also demonstrated that berberine inhibits the levels of cyclin

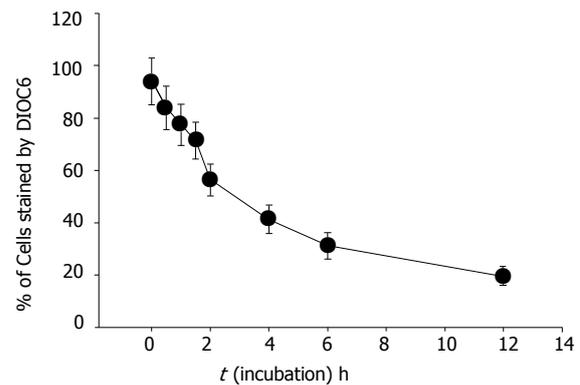


Figure 5 Flow cytometric analysis of mitochondrial membrane potential in human SNU-5 cells with 100 μ mol/L berberine for various time periods. The SNU-5 cells (5×10^5 cells/L) were treated with various concentrations of berberine. The zero concentration was defined as control. The percentage of cells stained with DiOC₆ dye, was determined by flow cytometry as described in the Materials and Methods.

B and CDK1 but increases the levels of Wee1 and Cdc2, which may be the factors for G₂/M arrest in SNU-5 cells. It has been reported that increases of Cdc2 activity are in response to drug-induced G₂/M arrest^[16,17]. Formation of Cdc2-cyclin B complex is necessary for G₂/M transition and cells to enter mitosis^[16]. Our result demonstrated that berberine declined cyclin b levels in SNU-5 cells, and that G₂/M arrest could be controlled by cyclin B rather than by Cdc2 activation. Cyclin-dependent kinases (Cdks) are the central regulators of cell division cycle. Inhibitors of Cdks ensure proper coordination of cell cycle events and regulate cell proliferation in tissues and organs. Wee1 homologs phosphorylate a conserved tyrosine to inhibit the mitotic cyclin-dependent kinase Cdk1^[18]. It was also reported that the induction of Cdc2 phosphorylation due to the increase of Wee1 and Myt1 as well as the reduction of Cdc2 and cyclin B1, is involved in 1,25 [OH] 2VD3-induced G₂/M arrest of keratinocytes^[16,19]. It has been shown that multisite phosphorylation of either CDK, Cdc2, Wee1, or CDK-activating kinase is sufficient to generate dynamical behaviors including bistability and limited cycles^[20,21]. Experimental depletion of Wee1 by a small interfering RNA directed to Wee1 mRNA could alleviate Vpr-induced G(2) arrest and allow normal progression from M into G phase^[16].

Cell cycle analysis revealed the presence of apoptotic cell death (sub-G₁ group) following treatment with berberine. We also did morphological examination which showed cell shrinkage, loss of cell-to-cell contact, membrane blebbing and chromatin condensation elicited by increasing berberine concentration and length of exposure. These results were also confirmed by fluorescence microscopy and flow cytometry. So far, many signals and stimuli have been reported to join the induction of apoptosis, therefore the survival of specific cells is under the control of a wide complex of signals. Especially the caspase activities have been demonstrated to be the regulators of apoptosis^[22,23]. Most apoptosis models are involved in caspase activation and two main pathways: caspases-8 and -3 or activation of caspases-9 and -3 activation^[24]. The caspases-9 and -3 are involved in the

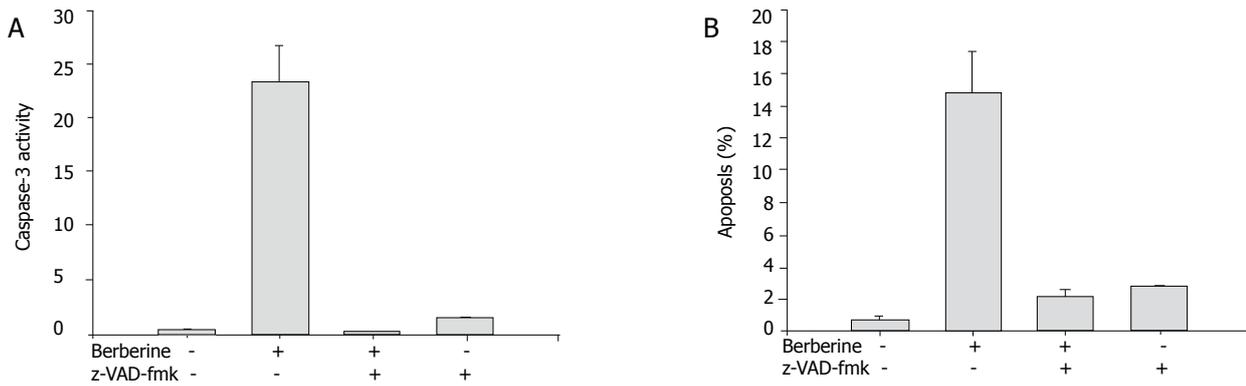


Figure 6 Flow cytometric analysis of the effects of berberine induced caspase-3 activity (A) and apoptosis (B). The SNU-5 cells were incubated with 100 $\mu\text{mol/L}$ berberine with or without z-VAD-fmk treatment for determination of caspase-3 activity and apoptosis.

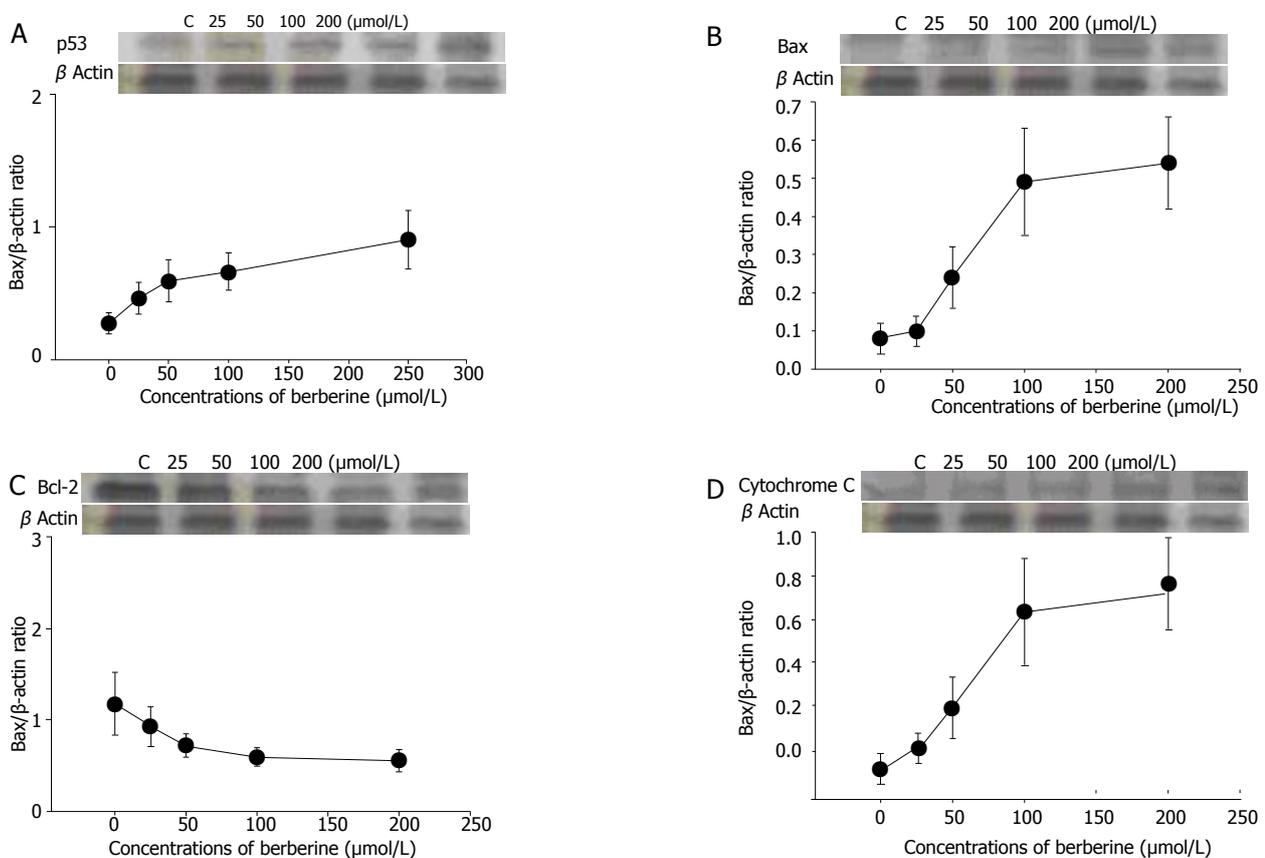


Figure 7 Changes of levels of p53(A), Bcl-2(B), Bax(C), and cytochrome C(D) in SNU-5 cells after exposure to berberine. SNU-5 cells ($5 \times 10^6/\text{mL}$) were treated with 0, 25, 50, 100, and 200 $\mu\text{mol/L}$ berberine for 24 h, then cytosolic fraction and total protein were determined as described in Materials and Methods. The levels of p53, p21, Bcl-2, Bax, and cytochrome C were determined by Western blotting as described in Materials and Methods.

release of cytochrome C from mitochondria which causes the decrease of mitochondrial membrane potential. Our data demonstrated that berberine was able to induce the increase of Ca^{2+} and mitochondrial membrane potential loss and cytochrome C release in cytosolic fraction of SNU-5 cells, which correlates well with the activation of caspases-9 and -3. Our result also showed that berberine decreased the levels of Bcl-2 which control mitochondrial membrane potential and Bax was increased which promotes the cytochrome C release (Figure 8).

Berberine can decrease apoptosis induced by paclitaxel

in human cancer cell lines including gastric cancer cells^[25,26], whereas in the present study berberine induced cell cycle arrest and cell death (apoptosis) in SNU-5 cells in a dose- and time-dependent manner. Based on these findings, we suggest that berberine may contribute to the antineoplastic activity of gastric cancer cells. However, the molecular basis of such effects needs for further investigations. Although the exact binding sites or receptors of berberine on the SNU-5 cells are still unknown, the antineoplastic activity of berberine may provide a basis for further studies.

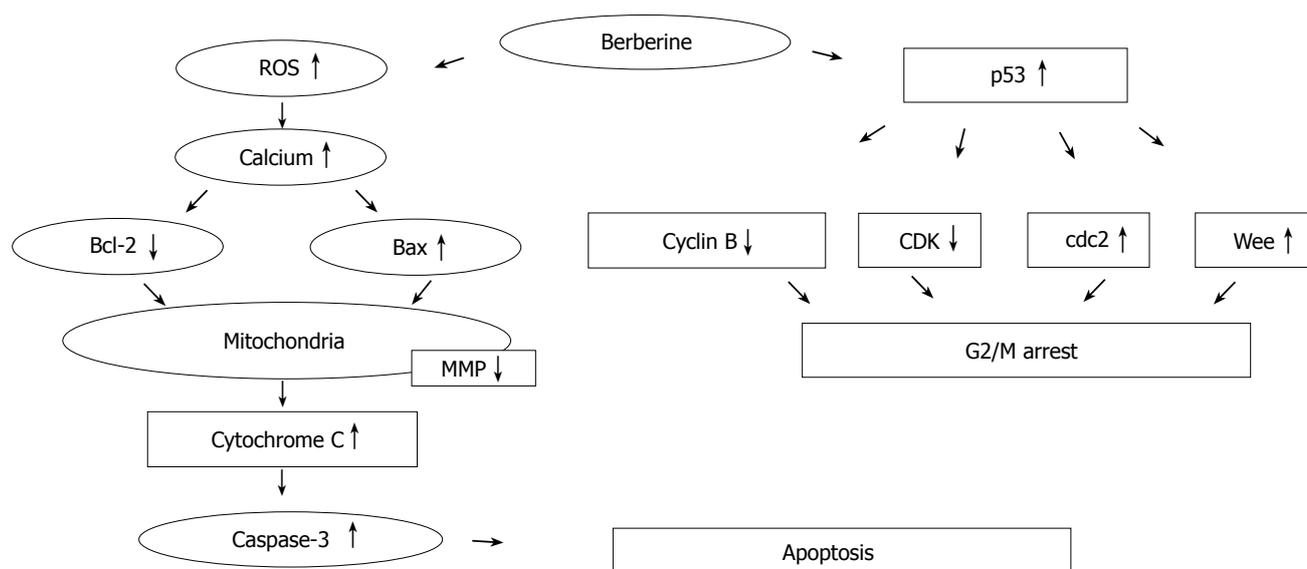


Figure 8 Proposed model of berberine mechanism of action on G2/M arrest and apoptosis in SNU-5 cells. Berberine induced p53 expression that led to the decrease of cyclin B and CDK1 but increase of the expression of cdc25c and Wee1 for G2/M arrest. Berberine induced ROS, Ca²⁺ production and decreased MMP levels led to cytochrome C release and caspase-3 activity, causing apoptosis in SNU-5 cells.

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Inhibitory effect of Polo-like kinase 1 depletion on mitosis and apoptosis of gastric cancer cells

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Abstract

AIM: Polo-like kinase 1 (PLK1) serine/threonine kinase plays a vital role in multiple phases of mitosis in gastric cancer cells. To investigate the effect of PLK1 depletion on mitosis and apoptosis of gastric cancer cells.

METHODS: PLK1 expression was blocked by small RNA interference (siRNA). The expression levels of PLK1, cdc2, cyclin B and caspase 3 were detected by Western blotting. Then, PLK1 depletion, cdc2 activity, cell proliferation, cell cycle phase distribution, mitotic spindle structure, and the rate of apoptosis of the PLK1 knockdown cells were observed.

RESULTS: PLK1 gene knockdown was associated with increased cyclin B expression, increased cdc2 activity (but not with the expression levels), accumulation of gastric cancer cells at G2/M, improper mitotic spindle formation, delayed chromosome separation and delayed or arrested cytokinesis. Moreover, PLK1 depletion in gastric cancer cells was associated with decreased proliferation, attenuated pro-caspase 3 levels and increased apoptosis.

CONCLUSION: Blockage of PLK1 expression may lead to decreased mitosis or even apoptosis in gastric cancer cells, indicating that PLK1 may be a valuable therapeutic target for gastric cancer.

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Key words: Gastric cancer cells; PLK1 gene; Mitosis; Cell cycle; Apoptosis

INTRODUCTION

The separation of chromosomes and the division of one cell into two daughter cells are the most dramatic events during the cell cycle. This process, known as mitosis, involves a series of structural changes in cells, including centrosome duplication, spindle formation and cytokinesis. The cdc2-cyclinB protein complex, a mitosis promoting factor, is known to trigger and promote the completion of this complex process^[1], but the mechanism responsible for activating the cdc2-cyclinB complex remained unknown until the conserved Polo-like kinase 1 (PLK1) was cloned by Golsteyn *et al.*^[2]. PLK1 accumulates markedly during the G₂/M phase in cells, where it phosphorylates and activates the cdc2-cyclinB complex and other mitosis-involved substrates^[3,4]. PLK1 is indispensable in some types of cell division including tumor cells^[5]. Besides, PLK1 expression increases in multiple types of solid tumors such as non-small-cell lung cancer, head and neck cancer, esophageal cancer, colorectal cancer-associated glioma^[6-10]. PLK1 is now considered as an oncogene, a potential target for cancer therapy. But to our knowledge, no previous work has examined the effect of PLK1 knockdown in gastric cancer cells. Here, we have used RNA interference technology to block the expression of PLK1 in gastric cancer cell line MKN45 and observed the changes in cell division phenotypes and cell viability.

MATERIALS AND METHODS

Cell culture

The gastric cancer cell line MKN45 was maintained in RPMI1640 medium containing 2 mmol/L glutamine and 100ml/L fetal bovine serum (Invitrogen, CA, USA) and incubated at 37°C in an atmosphere containing 50 mL/L of CO₂.

PLK1 siRNA

The Ambion software was used to design RNAi sequences targeting human PLK1 (accession no. NM 005030) and

the siRNA sequence with the highest putative efficacy (5'-CAACCAAAGTCGAATATGA 3') was synthesized by Shanghai GeneChem Co., Ltd. (PLK⁻ group). Small RNA interference with randomized sequence (5'-TTCTCCGAACGTGTCACGT3') against no gene (scrambled siRNA group) and only liposome (liposome group) were transfected as internal control.

For the experiments, cells transfected with the DOTAP liposomal transfection reagent (Roche, Germany) were seeded at 2×10^5 cells/well in six-well plates. After 24 h culture when cells were in the phase of log growth, 250 μ L Opti-MEM I was mixed with 7.5 μ L of 20 μ mol/L siRNA duplex, while another 250 μ L Opti-MEM I was separately incubated with 11.88 μ L of DOTAP liposome. The two mixtures were gently mixed and incubated for about 30 min at room temperature. For transfection, the entire mixture was added to each well in 1.5 mL of fresh medium containing 100 ml/L FBS without antibiotics. The final transfected concentration of siRNA was 75 nmol/L. Cells were collected for further assay at 24, 48, and 72 h after transfection.

Western blotting

Cells were lysed in AM1 lysis buffer (Active Motif, USA) and protein concentrations were measured with the BCA protein assay kit (Pierce, USA). Total protein (50 μ g) was resolved by 125 g/L SDS-PAGE and transferred onto PVDF membranes. After being blocked in TBST (20 mmol/L Tris, 137 mmol/L NaCl, 1 g/L Tween 20, pH 7.6) with 50 ml/L skim milk for 2 h at room temperature, membranes were incubated with PLK1, cyclinB, cdc2, procaspase 3, and β -actin primary antibodies (diluted 1:200; Santa Cruz Biotechnology, USA) for 2 h. Membranes were then washed thrice with TBST solution, followed by incubation for 1 h with HRP-linked secondary antibodies (1:1 000; Santa Cruz Biotechnology) at room temperature. Finally, membranes were visualized using the DAB reagent (Dako Corporation, Denmark).

Kinase analysis

Cdc2 kinase activity was measured using the cdc2-cyclinB kinase assay kit (MBLTM International, Japan) according to the manufacturer's instructions.

Fluorescence-activated cell sorting analysis

Cells were harvested by trypsin, washed with cold PBS and resuspended in 750 ml/L ethanol at 4°C for at least 8 h. The fixed cells were collected by brief centrifugation and resuspended in PBS containing 200 μ g/mL RNase and 15 mg/L propidium iodide. After incubation at room temperature for 30 min, samples were subjected to flow cytometry (FCM) for Fluorescence-activated cell sorting assay and cell cycle phase analysis.

Immunofluorescence staining and confocal microscopy

Cells were grown on coverslips, fixed with 40g/L paraformaldehyde for 10 min and permeabilized with methanol for 2 min. After being washed thrice with 1 Triton X-100 in PBS, the coverslips were blocked with 20 ml/L FBS for 30 min, stained with 10 mg/L anti- α -

tubulin primary antibody for 2 h at room temperature and incubated with FITC-conjugated secondary antibody (Santa Cruz Biotechnology) for 30 min. Finally, the DNA was stained with TO-PRO-3 iodide (Molecular Probes, USA) and confocal microscopy was performed to obtain detailed images of the subcellular structures.

MTT assay

The MTT method was used to measure cell proliferation. Briefly, 4×10^3 cells/well were cultured in 96-well plates. After 24 h, siRNA and liposome were added to transfect cells, and then at the designated time points (24, 48, 72, and 96 h after transfection), MTT (5 g/L) was added. Cells were incubated for 4 h and then the medium was removed and 100 μ L DMSO solution was added for 5 min. The absorbance of the reaction solution was measured at 570 nm and the data from the various time points were used to generate cell proliferation curves.

Apoptosis detection

Apoptosis was assessed with an Annexin V kit (BD Corporation, USA). In brief, cells were harvested, washed in PBS and labeled with Annexin V antibody and PI according to the manufacturer's protocol. Labeled cells were then subjected to FCM for the determination of apoptosis.

Statistical analysis

All experiments were repeated thrice. Data were analyzed with SPSS software version 11.0. Student's *t*-test was used to evaluate the difference in cell phase distribution between PLK⁻ and scrambled siRNA groups. One-way analysis of variance (ANOVA) was used to test the effects of the three treatments on cdc2 activity and apoptosis rate. Two-way analysis of variance was performed to detect the random effects of the three treatments on cell proliferation (MTT assay), and Student-Newman-Keuls test was used to detect the difference between any two groups. The χ^2 test was employed to examine the difference in cell mitosis phenotypes under confocal microscope. Cell morphology was observed under inverted microscope between experimental and control groups. $P < 0.05$ was considered statistically significant.

RESULTS

Increase in cyclinB expression and cdc2 activity following PLK1 knockdown by siRNA

To determine the effect of siRNA on PLK1 depletion, we collected cell samples at 24, 48, and 72 h after transfection, extracted total cellular proteins and performed standard Western blotting of experimental cultures and scrambled siRNA-treated and liposome-only controls. PLK1 protein levels were reduced by 38.4% and 60.7% compared with the scrambled siRNA-treated control groups at 48 and 72 h, respectively, indicating that PLK1 gene expression was obviously blocked by the siRNAs (Figure 1A). We found that the cyclinB protein levels were 82.4% and 32.9% higher at 48 and 72 h, respectively in PLK1-depleted

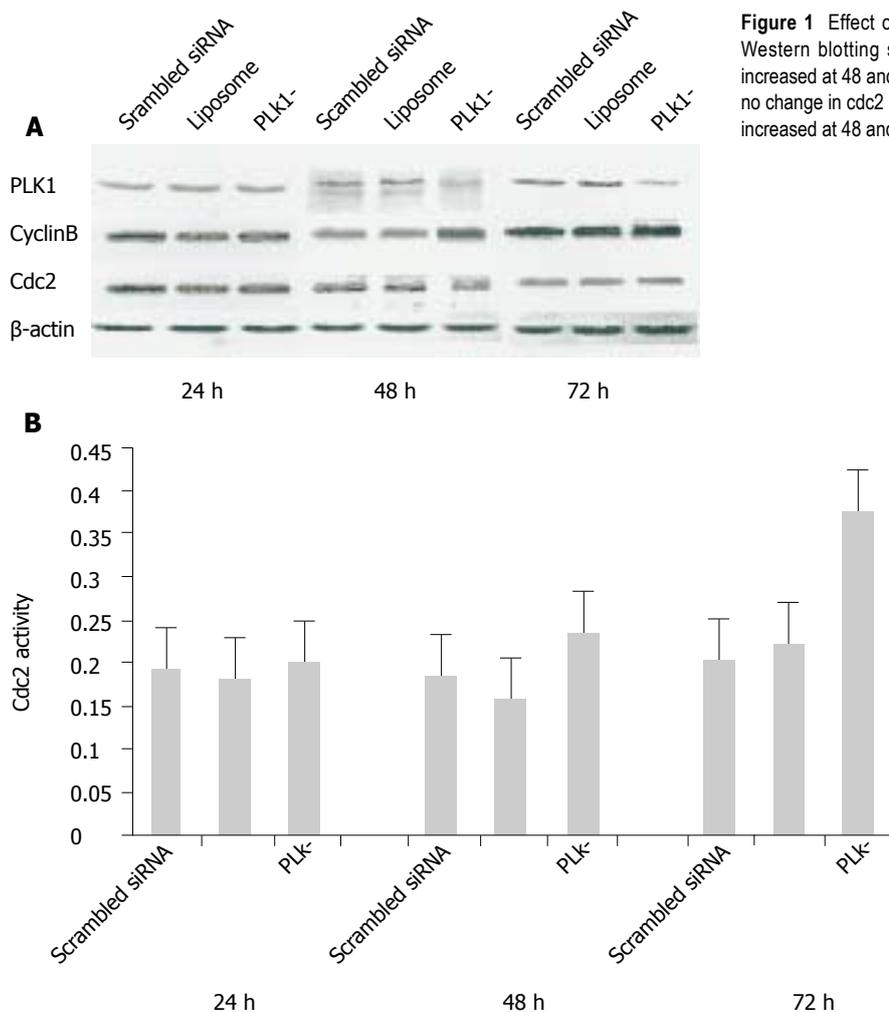


Figure 1 Effect of PLK1 depletion on members of the MPF complex. **A:** Western blotting showed that PLK1 levels decreased and cyclinB level increased at 48 and 72 h after RNAi targeting PLK, whereas there was almost no change in cdc2 level at 24, 48, and 72 h. **B:** Cdc2 activity was substantially increased at 48 and 72 h after PLK1 expression knockdown.

cells than in scrambled siRNA-treated cells (Figure 1A), while the mean activity of cdc2 kinase was increased in comparison to the controls (85.71% and 68% higher than scrambled siRNA-treated cells at 48 and 72 h, respectively, $P < 0.05$), but there was no apparent difference at the protein level (Figures 1A and 1B).

Tumor cell accumulation in G₂/M phase caused by PLK1 siRNA

Cell cycle phase distribution was measured by FCM at 24, 48, and 72 h after siRNA transfection. The mean percentage of cells with G₂ DNA content in the PLK⁻ group was 40.15%, 36.58%, and 59.88%, while that in the scrambled siRNA control group was 14.59%, 10.11%, and 17.69% at 24, 48, and 72 h, respectively ($P < 0.05$, Figure 2A). Inverted microscopy was used to examine the morphological changes of tumor cells under five randomly chosen inverted microscopic fields. The percentage of rounded cells was higher in the experimental group than in the scrambled siRNA control group (41% *vs* 13%, 38.55% *vs* 13.21%, and 44.55% *vs* 18.2% at 24, 48, and 72 h, respectively) ($P < 0.05$, Figure 2B).

Decreased tumor cell proliferation caused by PLK1 siRNA

MTT assays were used to determine whether PLK1 gene depletion affected tumor cell proliferation. The resultant

proliferation curves indicated that the PLK1-depleted cells divided more slowly than cells in the two control groups ($P < 0.05$, Figure 3).

Spindle formation, chromosome separation and cytokinesis in gastric cancer cells delayed by PLK1 siRNA

Changes in the mitotic phenotypes of PLK1 knockdown and control cells were observed by immunofluorescence staining and confocal microscopy. The spindle structure lost its cohesiveness 48 h after siRNA transfection (Figure 4A). Images of the five different substages of mitosis could be acquired (Figure 4B). There were substantial differences in the amounts and percentages of cells between the experimental group and scrambled siRNA control group after 48 h ($P < 0.05$, Table 1) at the onset of mitosis. More PLK⁻ cells (46% *vs* 20%) were at substage I (nuclear membrane breakdown and even chromosomal distribution in the cytoplasm) and fewer (1% *vs* 41%) were at substage II (chromosomal array along the equator plate) and III (2% *vs* 8%) (chromosomal segregation). Meanwhile, higher percentages of cells with dumbbell-shaped nuclei (33% *vs* 15%) and cytoplasmic bridges connecting two incompletely separated cells (8% *vs* 1%) were also shown in PLK⁻ cells. These results collectively indicated that spindle formation, chromosome separation

Table 1 Effect of PLK1-specific siRNA on MKN45 mitosis phenotype¹ (%)

| | I | II | III | IV | V | Other types | Total |
|------------------|----|----|-----|----|---|-------------|-------|
| Scrambled siRNA | 20 | 41 | 8 | 15 | 1 | 15 | 100 |
| PLK ⁻ | 46 | 1 | 2 | 33 | 8 | 10 | 100 |

¹314 mitosis cells in control group and 233 mitosis cells in RNAi group were counted.

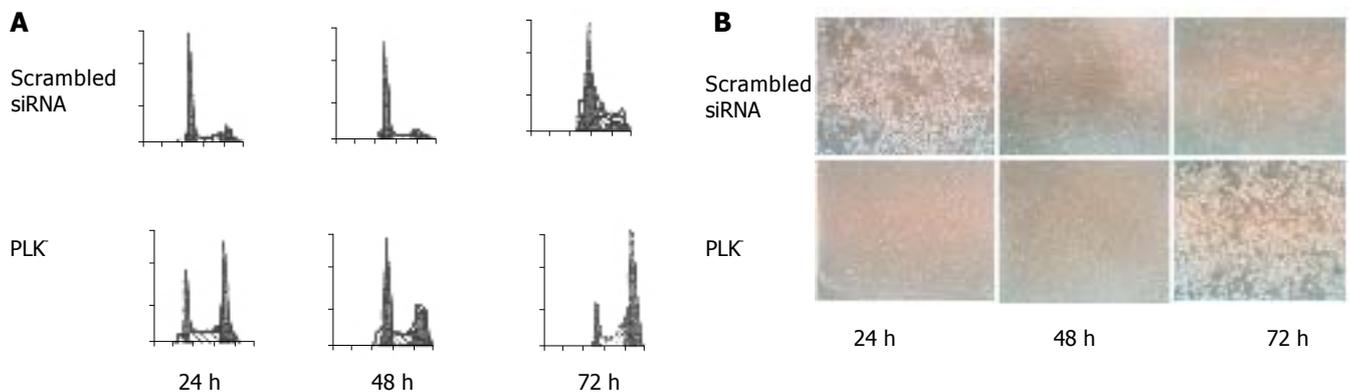


Figure 2 Changes in tumor cell cycle after PLK1 depletion. **A:** Fluorescence-activated cell sorting (FACS) analysis revealed that PLK⁻ MKN45 cells had an increased G₂ DNA content and were accumulated at G₂/M phase. **B:** Cells treated with siRNA became rounder in shape.

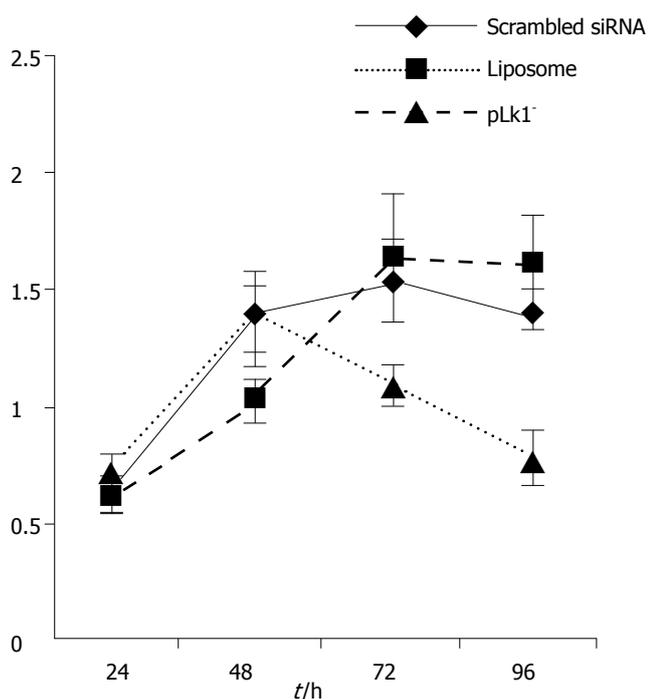


Figure 3 Effect of PLK1 depletion on cell proliferation.

and cytokinesis were delayed during mitosis in MKN45 cells transfected with PLK1-specific siRNA duplexes.

Increased apoptosis of MKN45 cells caused by PLK1 knockdown

To evaluate the role of PLK1 in tumor cell fate, PLK1-depleted cells were labeled with Annexin V antibody and subjected to FCM. The results showed that the mean apoptosis rate (including early and late apoptosis) at 48 and 72 h was higher in PLK1-depleted cells than in scrambled

siRNA cells (42.4% *vs* 21.4%, 53.8% *vs* 32.9%, $P < 0.05$, Figure 5A). Furthermore, Western blotting revealed that caspase 3 level was lower in PLK1-depleted cells (Figure 5B).

DISCUSSION

Gastric cancer is one of the most common malignancies in the world. Uncontrolled cellular proliferation is commonly associated with the poor prognosis of this disease. Human PLK1 plays a key role in some mitotic events^[11,12]. Its expression is associated with tumor proliferation degree^[13]. PLK1 depletion is associated with HeLa cell mitosis^[5]. However, no previous work has examined the possible role of PLK1 in gastric cancer cell mitosis and cell fate. In this experiment, we have used siRNA technology to knockdown PLK1 gene expression and observed the changes in mitotic phenotypes of the gastric cancer cell line, MKN45.

The protein level of PLK1 was decreased by 38.4% and 60.7%, respectively at 48 and 72 h after the addition of the PLK1-specific siRNA, indicating that the interference is effective and this system is appropriate for the study of mitotic events under PLK1 knockdown conditions. A previous study has demonstrated that the direct motivation of driving cells from G₂ to M phase is caused by the cyclinB-cdc2 complex (also known as the mitosis promoting factor or MPF). PLK1 is responsible for phosphorylating and activating MPF at the G₂/M checkpoint^[3] and mediates the degradation of cyclinB by activating the anaphase-promoting complex at the end of mitosis^[14,15], indicating that PLK1 can initiate and maintain mitosis. In our PLK1 knockdown cells, the cyclinB level increased probably due to the failure of depleted PLK1

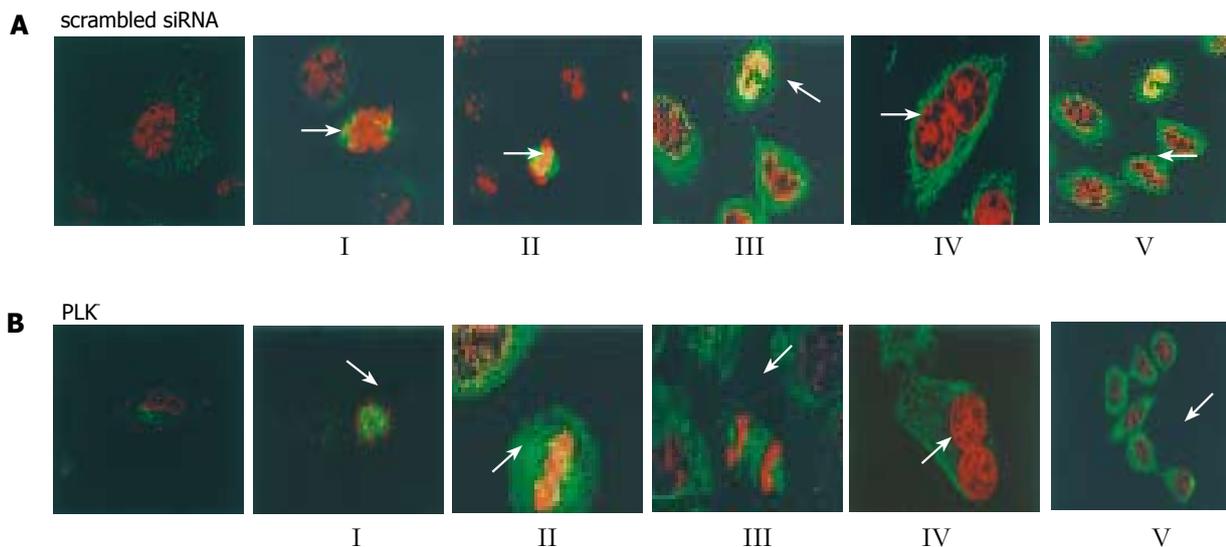


Figure 4 Tumor cell mitosis decreases following PLK1 depletion. **A:** Control cells showed clear mitotic frameworks, whereas the spindles of the PLK¹ depleted cells were unclear and broken when the sister chromosomes were separated. **B:** The five examined substages of mitosis (I-V) in both control and PLK¹ depleted cells.

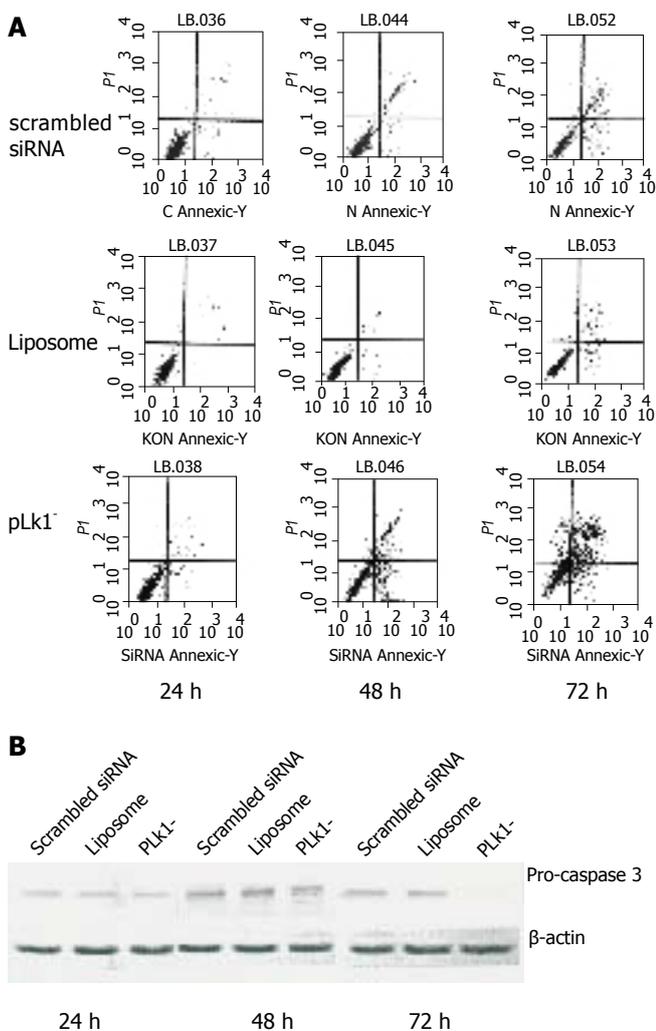


Figure 5 PLK1 siRNA induces MKN45 apoptosis. **A:** Annexin V staining and FCM revealed a higher percentage of apoptotic PLK1-depleted cells at 48 and 72 h; **B:** Western blotting showed reduced pro-caspase 3 levels in PLK¹ cells at 48 and 72 h.

quantity) of *cdc2* (a component of MPF) was increased by 85.7% and 68.0% at 48 and 72 h, respectively in PLK1 knockdown cells compared to scrambled siRNA controls. Previous reports indicate that PLK1 and its analogs seem to phosphorylate and activate *cdc25*^[3,16], which then dephosphorylates and activates the *cdc2*/cyclinB complex in different organisms^[17,18], suggesting that *cdc2* may lose its activity under PLK1 knockdown conditions. Our observation of *cdc2* activation seems to indicate that *cdc2* is activated in a *cdc25*-independent manner.

Immunofluorescence and confocal microscopy revealed that the microtubule morphology was affected in PLK1-depleted cells, becoming broken and unclear. We were able to identify cells in both PLK1-depleted and control cultures corresponding to the five substages of mitosis: (I) nuclear membrane breakdown and even chromosomal distribution in cytoplasm; (II) chromosomal array along the equator plate; (III) chromosomal segregation; (IV) mitotic exit and nuclear membrane formation; (V) cytokinesis. These substages were observed at varying frequencies between the control and siRNA-treated cells. At the onset of mitosis, more siRNA-treated cells (46% *vs* 20%) were at substage I and fewer were at substage II (1% *vs* 41%) or III (2% *vs* 8%). Recent studies showed that PLK1 affects chromosomal separation by controlling the formation of mitotic spindle and phosphorylating cohesion to decrease the cohesion of sister chromosomes^[19-21]. Our results revealed that in PLK1-depleted tumor cells, the mitotic spindle was disrupted (Figure 4A), which may be the reason why many PLK1-depleted cells are stalled in substage I and unable to progress to the subsequent stages requiring intact mitotic spindles.

In addition to its effect on chromosomal segregation, PLK1 is also closely associated with the exit from mitosis and subsequent cytokinesis. In the former, PLK1 appears to play a role in the activation of APC by destroying the APC inhibitor, Emi1^[15,22]. In cytokinesis, PLK1 is associated with the phosphorylation and activation of motor-like protein (MKlp) 2^[23] and nuclear distribution

to activate APC. In addition, the activity (but not the

gene C (NudC)^[24,25]. In our study, 48 h after siRNA treatment, α -tubulin immunofluorescence and DNA staining revealed a higher percentage of dumbbell-shaped cell nucleoli (33% *vs* 15%) and increased the number of cytoplasmic bridges connecting two incompletely separated cells (8% *vs* 1%), indicating that PLK1-depleted cells cannot successfully exit mitosis and reform two new nuclear membranes at anaphase but arrest mitosis during cytokinesis. In our study, immunohistochemistry and FCM experiments showed that the expression of PLK1-specific RNAi affected mitotic processes, causing tumor cells to accumulate at the mitotic phase and blocking them from completing cell division. In addition, our MTT assays indicated that PLK1-depleted cells decreased their proliferation.

We examined whether PLK1 depletion affected the fate of MKN45 cells. We used Annexin V staining to identify early and late apoptosis. Our results revealed that apoptosis increased significantly 48 and 72 h after the addition of the PLK1-specific siRNA. There was no obvious change at 24 h, indicating a delay before the apoptotic mechanism is triggered. Further examination revealed a sharp decrease in pro-caspase 3 levels. PLK1 can bind to and suppress the pro-apoptotic molecule p53 in HeLa cells^[26], while p53 is accumulated during PLK1-depletion-induced apoptosis^[27]. Based on this, it may be interesting to clarify whether any other pathway is involved in PLK1 depletion-induced apoptosis.

In conclusion, PLK1 is vital to gastric cancer cell division. Gene or drug therapy aimed at depleting PLK1 level may have a potential value as a novel treatment for gastric cancer.

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GASTRIC CANCER

CD44v6 in peripheral blood and bone marrow as micro-metastasis of patients with gastric cancer

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Abstract

AIM: To detect the expression of CD44 correlated with the ability of micro-metastasis in peripheral blood and bone marrow of patients with gastric cancer and to deduce its clinical significance.

METHODS: Preoperative peripheral blood and bone marrow specimens from 46 patients with gastric cancer and 6 controls were studied by semi-quantitative RT-PCR amplification of CD44v6mRNA. Preoperative and postoperative peripheral blood specimens from 40 patients with gastric cancer and 14 controls were studied by quantitative RT-PCR amplification of CD44v6mRNA in the corresponding period.

RESULTS: Semi-quantitative RT-PCR amplification showed that CD44v6mRNA expression of peripheral blood and bone marrow was positive in 39 (84.8%) and 40 (86.9%) of 46 patients with gastric cancer, respectively. In peripheral blood, CD44v6mRNA expression was positive for diffuse type in 30 (93.8%) of 32 patients and for intestinal type in 9 (64.3%) of 14 patients. On the other hand, in bone marrow, CD44v6mRNA expression was positive for diffuse type in 31 (96.9%) of 32 patients and for intestinal type in 10 (71.4%) of 14 patients. There was a significant difference between the diffuse type and intestinal type. Quantitative RT-PCR amplification demonstrated that CD44v6mRNA was not expressed in the peripheral blood of controls and CD44v6mRNA expression was positive for preoperative peripheral blood in 40 patients with gastric cancer, the expression levels being from 4.9×10^8 - 3.2×10^{11} copies/g RNA. The average expression level of CD44v6mRNA in peripheral blood was 3.9×10^{10}

copies/g RNA. The expression levels of CD44v6mRNA in peripheral blood in gastric cancer patients after curative operation increased from 5.5×10^6 - 7.6×10^9 copies/g RNA and the average level was 2.4×10^8 copies/g RNA (Figure 3B) ($P=0.00496$). After curative operation, the expression level decreased markedly.

CONCLUSION: Semi-quantitative and quantitative RT-PCR amplification for CD44v6mRNA is a sensitive and specific method for the detection of micro-metastasis in peripheral blood and bone marrow, which might be used as an indicator of tumor burden and therapeutic effect.

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Key words: Gastric cancer; Micro-metastasis; Peripheral blood; Bone marrow; CD44v6

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INTRODUCTION

Micro-metastasis cannot be detected by a general clinical pathological method. Gastric cancer is one of the most frequent malignant tumors. Tumor invasion, metastasis, and relapse are correlated with the prognoses of gastric cancer. Tumors metastasize through lymph and blood circulation. Twenty percent of patients have micro-metastasis^[1]. Tumor cell micro-metastasis in lymphocytes, peripheral blood, bone marrow, and abdominal cavity is the main reason of metastasis and relapse^[2,3]. Discovering gastric cancer in time is not only important for predicting relapse and prognoses, but also important for making decisions concerning therapy. With the development of molecular biology technology, the method of inspecting micro-metastasis has become more reliable^[4]. Semi-quantitative RT-PCR can reveal gastric cancer cells in peripheral blood and bone marrow^[5,6]. CD44v6, a highly glycosylated cell surface protein, is involved in cell-cell and cell-matrix interactions and takes part in cell motility, tumor growth, and invasion^[7].

CD44 is an integral membrane glycoprotein with an apparent molecular mass ranging 85-250 ku. It is

Table 1 Oligonucleotide primer and probe sequences used

| Gene | Oligonucleotide | Location sequence | PCR product | bp |
|----------------|-----------------|-----------------------------|-------------|----|
| CD44v | Upper primer | 5'-TCCAGGCAACTCCTAGTAGT-3' | 740 | |
| | Lower primer | 5'-CAGCTGTCCTGTGTGCGAA-3' | | |
| β -Actin | Upper primer | 5'-CTACAATGAGCTGCGTGTGGC-3' | 206 | |
| | Lower primer | 5'-CAGGTCCAGACGCAGGATGGC-3' | | |
| Probe | | 5'-TGAGATTGGGTTGAAGAAATC-3' | | |

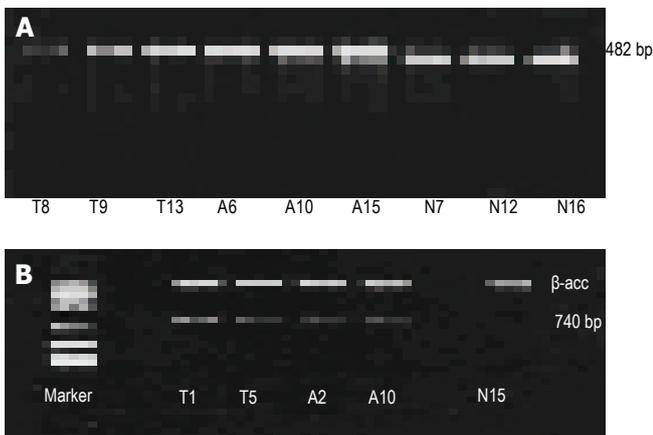


Figure 1 Expression of CD44s (A) and CD44v (B) in gastric mucosa. T1, T5, T8, T9, T13: bone marrow from patients with gastric cancer; A2, A6, A10, A15: peripheral blood from patients with gastric cancer; N7, N12, N15, N16: Controls.

originally described as a lymphocyte homing receptor on circulating lymphocytes^[8]. At least 20 variants (v) of CD44 have been reported^[9,10] due to the alternative splicing of 10 exons (v₁-v₁₀) that encode the membrane proximal portion of the extracellular domain. It has been reported that the expression of variant 6 of CD44 is correlated with invasion and metastasis of certain types of human cancer^[11]. The expression of CD44v6 and CD44v5 is correlated with tumor progression, metastasis, and prognosis of colorectal cancer, breast cancer, and gastric cancer.

In our study, we have also determined the expression of CD44v6mRNA using semi-quantitative RT-PCR in peripheral blood and bone marrow from 46 patients with gastric cancer. We have also determined the quantitative expression of CD44v6mRNA in peripheral blood specimens from 40 patients with gastric cancer and 14 controls using quantitative RT-PCR to display the role of CD44v6mRNA in clinical stage and prognosis of gastric cancer.

MATERIALS AND METHODS

Patients and serum samples

Eighty-six patients were randomly divided into gastric cancer group (52 male and 34 female patients with an average age of 58.6 years, ranging 32-81 years) and control group (14 male and 8 female patients with an average age of 53.8 years, ranging 40-65 years).

Serum samples were obtained from 86 patients with primary gastric cancer prior to surgery at the Department of Gastroenterology, the First Affiliated Hospital

of Yangzhou Medical University. The diagnosis was confirmed before surgery.

Extraction of total RNA

Before surgery, serum samples were obtained from peripheral blood and bone marrow 1 d before surgery and 9 d after surgery. Single nucleated cells were separated and stored at -20 °C. Total RNA was extracted. After being centrifuged at 2 500 r/min for 10 min, 5 mL S-ACR was added and bathed on ice for 15 min, then the process was repeated and the samples were stored at -70 °C.

cDNA synthesis, cDNA amplification, and semi-quantitative analysis

Primers sp1 and sp2 were from the cDNA sequence^[12]. Primers p1, p2, and β -actin were from the cDNA sequence^[13]. Primers were separately aimed at the standard and variant CD44s, CD44v6, and β -actin^[14]. The three couples of primers were synthesized by Sagon Co., Canada and stored at -20 °C (sp1: 5' -GACACATATTGCTTCAATG CTTCAGC-3'; sp2: 5' -GATGCCAAGATGATCAGCCATTCTGGAAT-3'; P1: 5'-GACAGACACCTCAGTTTTTCTGGA-3'; P2: 5'-TTC CTTCGTGTGTGGGTAATGAGA-3'; forward β -actin: 5' -CTACAATGAGCTGCGTGTGGC-3'; backward β -actin: 5'-CAGGTCCAGACGCA GGATGGC-3'). After cDNA was synthesized and amplified, the product was analyzed and the density was scanned (semi-quantitative analysis). When the value of CD44v6mRNA/ β -actin was less than 0.30 and more than 0.70, it was expressed as (-), (+), and (+).

Real-time RT-PCR

The point characterized the reactions during cycling, when the PCR product was first detected. The product was accumulated after a fixed number of cycles. The higher the starting quantity of the target molecule, the earlier the significant increase in fluorescence was observed. The parameter C+ (threshold cycle) was defined as the fractional cycle number at which the fluorescence was generated. CD44v6 target message in unknown samples was quantified by measuring C+ and using a calibration curve to determine the starting target message. The precise amount of total RNA added into each reaction mix (based on absorbance) and its quality were difficult to assess. For each experimental sample, the amount of targets and endogenous reference were determined by the calibration curve. The target amount was then divided by the endogenous reference amount to obtain a normalized target value. The relative gene target expression was also normalized to healthy control serum sample. Each of the normalized target values was divided by the calibrator-normalized target value to generate the final relative expression.

Primers, probes, and PCR consumables

Primers and probes for the CD44v6 gene were chosen with the assistance of the computer programs Oligo 4.0 (National Bioscience) and Primer Express (Perkin-Elmer Applied Bio-systems). We conducted BLAST searches against dbEST and nr to confirm the total gene specificity

Table 2 Relation between CD44v6mRNA expression in bone marrow and biologic behavior of gastric cancer

| | Number of cases (n) | CD44v6mRNA/β-actin | | | | P value | |
|-----------------------|---------------------|--------------------|---|---|---|---------|--------|
| | | - | + | + | + | | |
| Lymph node metastasis | (+) Positive | 38 | 3 | 2 | 4 | 29 | 0.0161 |
| | (-) Negative | 8 | 4 | 1 | 1 | 3 | |
| Clinicopathology | I-II | 12 | 5 | 2 | 2 | 3 | 0.0007 |
| | III-IV | 34 | 1 | 2 | 3 | 28 | |
| Tissue type | D | 32 | 1 | 2 | 4 | 25 | 0.0003 |
| | I | 14 | 5 | 2 | 4 | 3 | |
| Tumor size | >5 cm | 24 | 3 | 2 | 6 | 13 | 0.4547 |
| | ≤5 cm | 22 | 3 | 3 | 4 | 12 | |

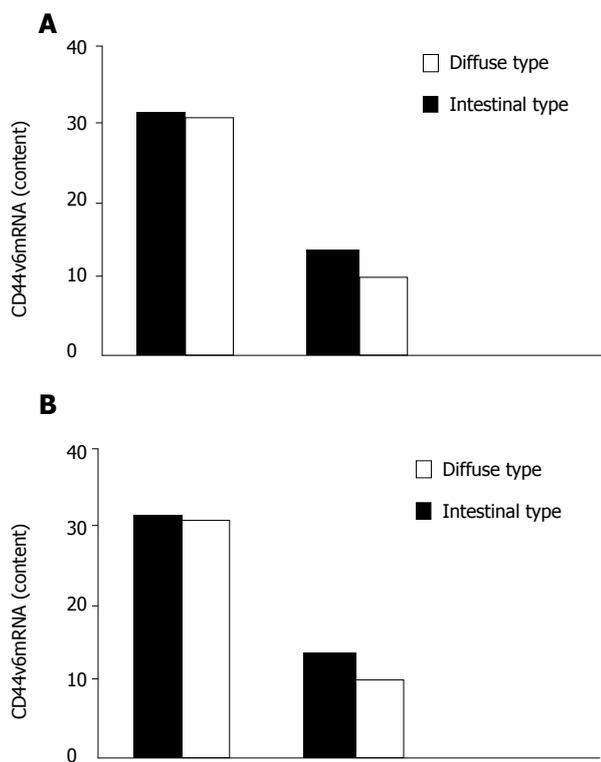


Figure 2 CD44v6mRNA expression in bone marrow (A) and peripheral blood (B) from patients with gastric cancer.

of the nucleotide sequences chosen for the primers and probes, and the absence of DNA polymorphisms. The primer for CD44v6 was selected and compared to the sequences of the closely related CD44v6 gene. The sequences of the oligonucleotide are shown in (Table 1). The primers and probes were designated by the nucleotide position corresponding to the 5'-position, followed by the letter U for upper (sense strand) or L for lower (antisense strand). To avoid amplification of contaminating genome DNA, one of the two primers or the probe was placed at the junction between two exons or in different exons.

RNA extraction

Total RNA was extracted from serum specimens of gastric cancer by the acid-phenol quantum method^[15]. The quality of RNA samples was determined by electrophoresis through denaturation of agarose gels and staining with

thallium bromide. The 18s and 28s RNA bands were visualized under ultraviolet light.

Calibration curve

Calibration curve was constructed with four fold serial dilutions of total RNA from healthy human serum. The diluted human total RNA was liquored and stored at -80 °C until use.

cDNA synthesis

Reverse transcription of RNA was performed in a final volume of 20 μL containing 1× RT-PCR buffer [500 mmol/L each dNTP, 3 mmol/L MgCl₂, 75 mmol/L KCl, 50 mmol/L Tris-HCl, pH 8.3, 10 μL of RNasin™ ribonuclease inhibitor (Promega), 10 mmol/L dithiothreitol, 50 μL of superscript RNase H⁻ reverse transcripts (Life Technologies), 1.5 mmol/L random hexanes and 1 μg of total RNA (calibration curve points and patient samples)]. The samples were incubated at 20 °C for 10 min and at 42 °C for 30 min. Reverse transcripts were inactivated by heating at 99 °C for 5 min and cooling at 5 °C for 5 min.

PCR amplification

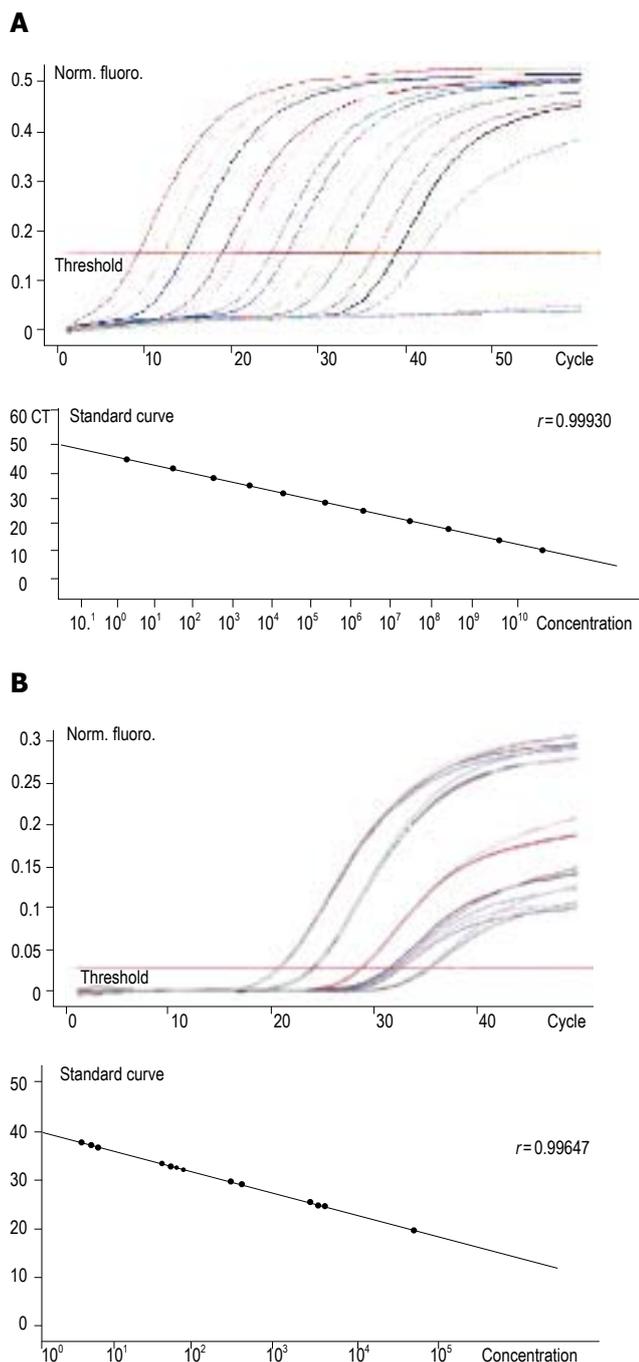
All PCR reactions were performed on an ABI PRISM 7700 Sequence Detection System. For each PCR, a master mixture was prepared on ice with 1× TaqMan buffer; 5 mmol/L MgCl₂; 200 mmol/L dATP, dCTP, and dGTP; 400 mmol/L dUTP; 300 mmol/L each primer; 150 mmol/L probe; 1.25 μL of Ampli Taq gold DNA. Polymers and 10 μL of each appropriately diluted reverse transcription sample were added to 40 μL of the PCR master mixture. The thermal cycling conditions were: an initial demodulation step at 95 °C for 10 min and 50 cycles at 95 °C for 15 s, at 65 °C for 1 min. Experiments were performed in duplicate for each data point. Each PCR run included five points of the calibration curve (fourfold serially diluted human normal gastric cDNA), a non-template control, cDNA calibrator. All patient samples with a cv of the number of CD44v6mRNA copies > 10% were retested.

Statistical analysis

The association of factors was evaluated by the χ^2 test. The significance of difference among the means was

Table 3 Relation between CD44v6mRNA expression in bone marrow and biologic behavior of gastric cancer

| | Number of cases (<i>n</i>) | CD44v6mRNA/actin | | | <i>P</i> value | |
|-----------------------|------------------------------|------------------|---|---|----------------|--------|
| | | - | + | + | | |
| Lymph node metastasis | (+) | 38 | 3 | 2 | 4 | 0.0131 |
| | (-) | 8 | 4 | 1 | 1 | |
| Clinicopathology | I-II | 12 | 5 | 2 | 2 | 0.0005 |
| | III-IV | 34 | 2 | 2 | 3 | |
| Tissue type | D | 32 | 2 | 2 | 4 | 0.0003 |
| | I | 14 | 5 | 2 | 4 | |
| Tumor size | >5 cm | 24 | 3 | 2 | 6 | 0.4240 |
| | ≤5 cm | 22 | 4 | 3 | 4 | |

**Figure 3** CD44v6mRNA expression in 20 peripheral blood samples from gastric cancer patients before (A) and after (B) surgery.

determined by the Student's *t* test and one-way analysis of variance. SPSS 11.0 for Windows 2000 was used. $P < 0.05$ was considered statistically significant.

RESULTS

Semi-quantitative analysis

Proliferation of CD44s in peripheral blood and bone marrow from patients with gastric cancer and controls is shown in Figure 1A.

CD44v6mRNA expression in bone marrow and peripheral blood was positive in 40 (86.9%) and 39 (84.8%) of 46 patients with gastric cancer. The positive CD44v expression was slightly higher in bone marrow than in peripheral blood ($P > 0.05$). The value of CD44v6mRNA expression was $64.6\% \pm 21.66\%$ (Figure 1B).

In 46 patients with gastric cancer, diffuse type was found in 32 cases and intestinal type in 14 cases. Positive CD44v6mRNA expression in bone marrow was found in 31 (96.9%) of 32 diffuse type patients and 10 (71.4%) of 14 intestinal type patients (Figure 2A). On the other hand, positive CD44v6mRNA expression in peripheral blood was found in 30 (93.8%) of 32 diffuse type patients and 9 (64.3%) of 14 intestinal type patients (Figure 2B). There was a significant difference between the diffuse and intestinal types.

The relation between CD44v6mRNA expression and pathological type, lymph node metastasis, clinical pathology, and the size of tumor is presented in Tables 2 and 3.

Calibration curve and dynamic range of real-time RT-PCR

The calibration curve was constructed from the total RNA extracted from healthy human gastric serum diluted fourfold in mouse total RNA. The primer chosen to analyze the CD44v6 gene did not amplify human genomic DNA or mouse cDNA. The dynamic range was at least three orders of magnitude in samples containing 50 μg or 0.2 ng equivalent to total cDNA. A strong linear relation between Ct and log of the starting copy number was demonstrated ($r^2 \geq 0.99$). The efficiency of the reaction (E) was 90%–100% calculated by the formula: $E = 101/[m] - 1$, where *m* is the slope of calibration curve.

To determine the cut-off value for altered CD44v6 gene expression at the RNA level in gastric cancer serum, the CD44v6 value (ratio of CD44v6mRNA to β -actin) was determined for four normal gastric serum RNAs. Because this value fluctuated between 0.5 and 1.7, values of 3 or more were considered as overexpression of the CD44v6 gene in tumor RNA samples.

CD44v6mRNA status and clinical and pathological factors

We sought for links between CD44v6mRNA status and standard clinical, pathological and biological factors in gastric cancer. Significant association was found between the overexpression of CD44v6mRNA gene and standard histopathological grade ($P < 0.05$) and negative progesterone receptor status ($P < 0.001$). A trend toward a link between the overexpression of CD44v6 gene and

Table 4 Quantitative expression of CD44v6mRNA in peripheral blood of patients with gastric cancer before and after surgery

| Id | Sex | Yr | Path | Metastasis | > 5 cm | Histopathology | Real-time RT-PCR ($\times 10^2$ copies/ μ g RNA) before surgery | Real-time RT-PCR ($\times 10^2$ copies/ μ g RNA) after surgery |
|----|-----|----|------|------------|--------|----------------|--|---|
| 1 | M | 56 | D | + | T | III | 3 100 | 300 |
| 2 | M | 52 | I | - | F | II | 4.9 | 2.0 |
| 3 | W | 60 | D | + | T | IV | 580 | 66 |
| 4 | W | 69 | D | + | T | III | 696 | 72 |
| 5 | W | 41 | D | + | T | IV | 3 200 | 460 |
| 6 | M | 39 | D | + | T | IV | 2 650 | 244 |
| 7 | M | 61 | I | - | F | II | 26 | 9.0 |
| 8 | W | 42 | D | + | T | IV | 360 | 48 |
| 9 | M | 71 | I | - | F | II | 590 | 72 |
| 10 | M | 61 | I | - | F | II | 430 | 58 |
| 11 | M | 41 | D | + | T | II | 1 230 | 142 |
| 12 | W | 47 | D | + | T | IV | 1 670 | 186 |
| 13 | M | 32 | D | + | T | IV | 1 340 | 166 |
| 14 | M | 49 | I | + | T | III | 280 | 36 |
| 15 | W | 61 | D | + | T | II | 1 080 | 128 |
| 16 | M | 76 | I | + | F | I | 480 | 68 |
| 17 | M | 81 | I | - | F | II | 36 | 6.9 |
| 18 | W | 60 | I | + | F | III | 290 | 42 |
| 19 | M | 37 | D | + | T | III | 980 | 112 |
| 20 | W | 40 | D | + | T | IV | 1 880 | 218 |
| 21 | W | 46 | D | + | T | III | 1 896 | 122 |
| 22 | W | 68 | I | - | T | IV | 2 208 | 342 |
| 23 | M | 72 | I | - | T | III | 1 960 | 140 |
| 24 | M | 64 | I | + | F | II | 868 | 22 |
| 25 | W | 51 | D | + | T | III | 1 020 | 100 |
| 26 | M | 32 | D | + | T | IV | 4 100 | 289 |
| 27 | W | 26 | D | + | T | IV | 3 908 | 432 |
| 28 | M | 72 | I | - | F | II | 680 | 107 |
| 29 | W | 74 | I | - | T | III | 1 024 | 210 |
| 30 | M | 61 | D | + | T | IV | 1 468 | 134 |
| 31 | M | 61 | I | | F | II | 430 | 101 |
| 32 | M | 41 | D | + | T | II | 1 230 | 11 |
| 33 | W | 47 | D | + | T | III | 1 670 | 25 |
| 34 | M | 32 | D | + | T | IV | 1 340 | 40 |
| 35 | M | 49 | I | + | T | I | 280 | 11 |
| 36 | W | 61 | D | + | T | II | 1 080 | 120 |
| 37 | M | 76 | I | + | F | I | 480 | 91 |
| 38 | M | 81 | I | - | F | I | 36 | 137 |
| 39 | W | 60 | I | + | F | II | 290 | 43 |
| 40 | M | 37 | D | + | T | III | 980 | 69 |

estrogen receptor negativity was also observed ($P=0.09$).

CD44v6mRNA amplification (patients for CD44v6mRNA expression)

As shown in Table 4, all the 20 peripheral blood samples of gastric cancer had the expression of CD44v6mRNA. The expression level ranged 4.9×10^8 - 3.2×10^{11} copies/g RNA and the average levels of peripheral blood was 3.9×10^{10} copies/g RNA (Figure 3A). The expression level of gastric cancer was 5.5×10^6 - 7.6×10^9 copies/g RNA and the average level was 2.4×10^8 copies/g RNA (Figure 3B) ($P=0.00496$) after curative surgery.

DISCUSSION

Gastric cancer is one of the most frequent malignant tumors. The survival rate of patients after radical surgery

of gastric cancer is 40% and patients always die because of metastasis and relapse^[16]. Some patients have already existed micro-metastasis which is not detectable by general clinical pathology during the treatment. The detection of micro-metastasis is correlated with the prognosis of gastric cancer patients^[17,18]. It was reported that micro-metastasis could be detected in lymph nodes of patients with intestinal cancer with negative pathology^[19]. Micro-metastasis has been considered as an indicator of prognosis and the value of micro-metastasis is superior to the Duke's stage and tumor grade. Zhang *et al.*^[20] demonstrated that tumor cells in metastatic lymph nodes of colorectal carcinoma possess cell proliferation activity and metastatic ability of tumor cells. Series slice examination has been used in inspecting tumor micro-metastasis since 1920s, but it is difficult to popularize^[21]. RT-PCR is a sensitive and specific method which can find

a tumor cell from 1×10^6 peripheral blood monocytes^[22]. Positive CD44v6mRNA expression was found in 39 of 46 patients with gastric cancer and negative CD44v6mRNA expression in patients with remote metastasis. The latter may be caused by sampling error^[23].

In our research, the peripheral blood and bone marrow specimens from 46 patients with gastric cancer and 6 controls were compared. The positive CD44v6mRNA expression rate was 84.4% and 86.9%, respectively in patients with gastric cancer. The CD44v6mRNA expression rate of diffuse type cancers was higher than that of intestinal type cancers, suggesting that the expression of CD44v6mRNA is correlated with the malignant phenotype of gastric cancer and CD44v6mRNA can be used as an indicator of the degree of tumor invasion and lymph node metastasis^[24]. The positive rate of CD44v6 is high in gastric cancer and may serve as a marker for diagnosing gastric cancer^[25-27].

The CD44v6mRNA expression rate in patients with lymph node or remote metastasis was higher than that in those without lymph node metastasis. The positive micro-metastasis rate of grades III-IV gastric cancer was significantly higher than that of grades I-II gastric cancer, suggesting that the expression of CD44v6mRNA can be used as an indicator of relapse and metastasis. CD44v6 expression is a significant risk factor for lymph node metastasis in patients with advanced carcinoma. Expression of CD44v6 plays an important role in tumor progression and may be a useful predictor of lymph node metastasis^[28-32].

The quantitative expression of CD44v6mRNA in 40 patients with gastric cancer before and after surgery showed that CD44v6mRNA was an indicator of tumor burden and therapeutic effect. The results showed that the expression level of CD44v6mRNA in peripheral blood of terminal gastric cancer patients (grades III-IV) was obviously higher than that in early gastric cancer patients (grades I-II). The expression level of CD44v6mRNA in gastric cancer patients with remote metastasis was obviously higher than that in those without metastasis. The expression level of CD44v6mRNA obviously decreased after curative surgery. The findings indicate that the expression level of CD44v6mRNA in peripheral blood is correlated with tumor burden.

Animal experiments indicate that 0.01% of tumor cells in circulation may lead to positive metastasis^[31,33,34]. Gulmann *et al.*^[35] showed that CD44v6 expression is a late phenomenon in the transformation of intestinal metaplasia to dysplasia/cancer. Gene expression of severe gastric mucosal dysplasia displays an obviously latent malignant tendency and gastric carcinoma with the expression of CD44v6 protein has a stronger ability to infiltrate and to metastasize via lymph nodes^[36]. Serum level of sCD44v6 could be taken as the criterion for evaluating the development and prognosis of gastric cancer, as well as the therapeutic target for anti-metastasis^[37]. Therefore, micro-metastasis should be routinely detected to afford evidence for establishing individual therapy scheme^[38]. Quantitative RT-PCR can improve the diagnostic sensitivity and specificity. Semi-quantitative RT-PCR is restricted because it cannot generate accurate gene quantification^[15]. In

our study, we have validated a RT-PCR method recently developed for the quantification of gene expression based on real-time analysis of PCR amplification and TaqMan methodology, which has several advantages over other RT-PCR-based quantitative assays such as competitive quantitative RT-PCR^[39].

Xin *et al.*^[40,41] discovered that patients with positive CD44v6 have a lower 3- and 5-year survival rate ($P = 0.0002$). Immunohistochemical detection of CD44v6 could now be used as an indicator of tumor progression in patients with gastric carcinoma. Tumor cells in the bone marrow indicate that patients have blood micro-metastasis^[42]. The probability of relapse and remote metastasis is great for patients who have postoperative bone marrow micro-metastasis and dynamic observation of micro-metastasis in these patients can predict the therapeutic effect and establish individual therapy scheme. Yamaguchi *et al.*^[43] found that CD44v6-positive cancers are more frequently associated with hematogenous metastasis. Therefore, blood is another metastatic route of gastric cancer. Detecting peripheral blood micro-metastasis of early gastric cancer patients is of great importance in predicting the prognosis and deciding the rational therapy of gastric cancer patients.

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Usefulness of endoscopic ultrasonography in preoperative TNM staging of gastric cancer

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Abstract

AIM: To evaluate the value of endoscopic ultrasonography (EUS) in the preoperative TNM staging of gastric cancer.

METHODS: Forty-one patients with gastric cancer (12 early stage and 29 advanced stage) proved by esophagogastroduodenoscopy and biopsies preoperatively evaluated with EUS according to TNM (1997) classification of International Union Contrele Cancer (UICC). Pentax EG-3630U/Hitachi EUB-525 echo endoscope with real-time ultrasound imaging linear scanning transducers (7.5 and 5.0 MHz) and Doppler information was used in the current study. EUS staging procedures for tumor depth of invasion (T stage) were performed according to the widely accepted five-layer structure of the gastric wall. All patients underwent surgery. Diagnostic accuracy of EUS for TNM staging of gastric cancer was determined by comparing preoperative EUS with subsequent postoperative histopathologic findings.

RESULTS: The overall diagnostic accuracy of EUS in preoperative determination of cancer depth of invasion was 68.3% (41/28) and 83.3% (12/10), 60% (20/12), 100% (5/5), 25% (4/1) for T1, T2, T3, and T4, respectively. The rates for overstaging and understaging were 24.4% (41/10), and 7.3% (41/3), respectively. EUS tended to overstage T criteria, and main reasons for overstaging were thickening of the gastric wall due to perifocal inflammatory change, and absence of serosal layer in certain areas of the stomach. The diagnostic accuracy of metastatic lymph node involvement or N staging of EUS was 100% (17/17) for N0 and 41.7% (24/10) for N+, respectively, and 66% (41/27) overall.

Misdiagnosing of the metastatic lymph nodes was related to the difficulty of distinguishing inflammatory lymph nodes from malignant lymph nodes, which imitate similar echo features. Predominant location and distribution of tumors in the stomach were in the antrum (20 patients), and the lesser curvature (17 patients), respectively. Three cases were found as surgically unresectable (T4 N+), and included as being correctly diagnosed by EUS.

CONCLUSION: EUS is a useful diagnostic method for preoperative staging of gastric cancer for T and N criteria. However, EUS evaluation of malignant lymph nodes is still unsatisfactory.

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Key words: Endoscopic ultrasonography; Preoperative staging; Gastric cancer

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INTRODUCTION

The incidence of gastric cancer is declining worldwide. However, it still remains the second most common cause of cancer-related death in the world^[1,2]. Typically, gastric cancer is asymptomatic when cancer is at early stage of disease; therefore, majority of patients present in advanced stage, and the mortality rate of this disease is still very high. The diagnosis of gastric cancer is based on esophagogastroduodenoscopy with biopsy following double-contrast x-ray examination. Presently, endoscopic ultrasonography is the most reliable nonsurgical method obtainable for assessing the primary tumor with high diagnostic rate of staging gastric cancer and lymph node involvement. EUS is also becoming a promising diagnostic modality for the evaluation of gastrointestinal submucosal tumors and large gastric folds^[3-7].

The complete treatment of gastric cancer is surgery, only tumor resection with involved lymph nodes associated with satisfactory prognosis. Survival after surgery is highly dependent on the stage of gastric cancer or anatomical extent of disease at the time of operation. Therefore, the accurate preoperative staging of gastric cancer is the most significant prognostic factor that predicts surgical outcome

Table 1 Relationship between EUS and anatomic layers of normal gastric wall

| EUS | Histology |
|-----------------------------------|--|
| 1 st hypoechoic layer | Water interface and superficial mucosa |
| 2 nd hypoechoic layer | Deeper mucosa |
| 3 rd hyperechoic layer | Submucosa |
| 4 th hypoechoic layer | Muscularis propria |
| 5 th hyperechoic layer | Serosa and subserosal fat |

Table 2 Correlation of UICC/AJCC classification for depth of primary esophageal or gastric cancer invasion (T) with EUS imaging for clinical staging^[9]

| Stage | EUS (abnormal) |
|------------------------------|------------------------------|
| T1-mucosa/submucosa | 1 st three layers |
| T2-muscularis propria | 4 th layer |
| T3-through adventitia/serosa | 5 th layer |
| T4-adjacent organ | Adjacent organ |

Table 3 Accuracy of EUS in preoperative stage determination of 41 patients with gastric cancer

| Histopathological T stage | n | EUS correct n/% | EUS over-staging n/% | EUS under-staging n/% |
|---------------------------|--------|-----------------|----------------------|----------------------------|
| PT1 | 12 | T1 10/83.3 | T2 2/16.7 | - |
| PT2 | 20 | T2 12/60 | T3 8/40 | - |
| PT3 | 5 | T3 5/100 | - | - |
| PT4 | 4 (3) | T4 1/25 | - | T2 2 (2)/50 T3 1 (1)/25 |
| Total | 41 (3) | 28/68.3 | 10/24.4 | 3 (3)/7.3 |

Three cases of unresectable T4 N+ tumors were correctly diagnosed.

and 5 years of survival and is essential for well-informed decisions on stage depending patient management to plan appropriate treatment. Such precise stage depending management will limit the occurrence of unnecessary exploratory surgical interventions^[8].

The aim of the present study was to evaluate the usefulness of EUS in TNM staging of stomach cancer comparing with postoperative histopathological findings.

MATERIALS AND METHODS

Patients

Between April 2001 and April 2004, 41 patients (29 men and 12 women; age range, 28 - 80 years; mean age 57 years) with gastric cancer diagnosed by EGD and confirmed with biopsy specimen, underwent EUS examination prior to surgery for tumor depth of invasion and lymph node involvement at our Department of Endoscopy. Twelve of them were in early gastric cancer stage and 29 were in advanced stage. All patients underwent surgery.

Apparatus and EUS examination procedures

The Pentax EG - 3630U/Hitachi EUB - 525 echo endoscope with real-time ultrasound imaging linear scanning transducers (7.5 and 5.0 MHz) and Doppler information was used in the present study. This echo endoscope also provides the instrument channel for performing fine-needle aspiration biopsy. On the tip of the endoscope, a balloon is placed which is filled by deaerated water for improved coupling of the ultrasound waves to the gastrointestinal wall by producing a fluid interface and displacing intraluminal air. Prior to each EUS, examination was performed by EGD with biopsy to confirm gastric cancer. After oropharyngeal local anesthesia, patients were examined in a left lateral position. The echo endoscope was advanced into the stomach, and the lesions were first examined endoscopically. Next the stomach was insufflated

Table 4 Accuracy of EUS in preoperative determination of N stage in 41 patients with gastric cancer

| Histopathological N stage | n | EUS correct n/% | EUS incorrect n/% |
|---------------------------|--------|-----------------|---------------------------|
| PN0 | 17 | N 17/100 | - |
| PN+ | 24 (3) | N+ 10 (3)/41.7 | N0 14/58.3 false negative |
| All cases | 41 (3) | 27 (3)/66 | 14/34 |

with 200 - 500 mL deaerated water and observed from the pylorus to the cardia by moving the tip of the endoscope for revealing cancer abnormalities and lymph nodes involvement. The findings were recorded at the computer database of our department and interpreted following a standard protocol with regard to tumor invasion according to the widely accepted five-layer structure of the gastric wall (Table 1).

The assessment of tumor invasion depth or T stage was defined as a hypoechoic structure alternating five-layer ultrasonographic structure of gastric wall. Tumors were staged according to TNM (1997) classification criteria of International Union contrele Cancer (UICC). T1 lesion was seen as a disruption of the first three layers (tumor invades the mucosa or submucosa). T2 lesion was seen as an invasion of the fourth layer (tumor invades the muscularis propria). T3 lesion was seen as a penetration through the fifth layer (tumor invasion of the serosa). T4 lesion was seen as an invasion of the adjacent organs and structures (Table 2). T1 stage showed EUS images of early gastric cancer, T2-T4 stages showed EUS images of advanced gastric cancer. Lymph nodes had round border and hypoechoic structures were considered as malignant. Stage N0 referred to no sign of metastasis. N+ referred to metastases in perigastric lymph nodes.

RESULTS

Findings of the 41 patients at preoperative EUS were postoperatively compared with histopathologic findings for T and N staging.

Surgical findings

Tumors were located in the fundus and cardia region ($n = 4$), in the body ($n = 11$), in the body and antrum ($n = 2$), in the antrum ($n = 20$) of the stomach, diffusely located ($n = 2$) and residual stomach ($n = 2$). Distribution of tumors was in the anterior wall ($n = 5$), posterior wall ($n = 5$), greater curvature ($n = 5$), and the lesser curvature ($n = 17$) of the stomach and 9 were circumferential. Three cases

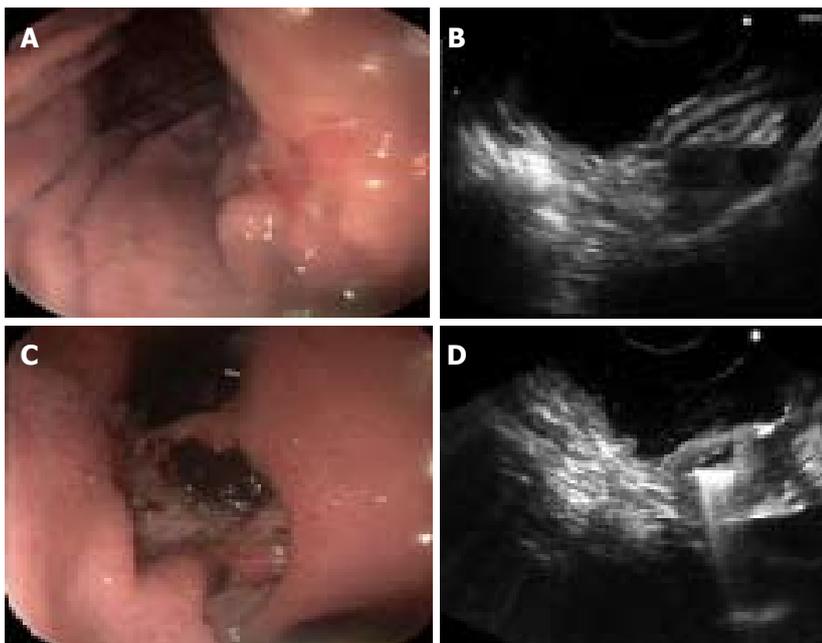


Figure 1 Early and advanced gastric cancer cases. **A:** Endoscopic view of superficial depressed type of early gastric cancer; **B:** EUS image shows cancer invasion of 1st and 2nd (mucosal) layers of gastric wall, while 3rd (submucosal) layer is clear (T1 category). Histopathological findings of the surgically resected specimen corresponded with the EUS findings; **C:** Endoscopic view of advanced Borrmann II type of gastric cancer; **D:** EUS images show disruption of 1-4 layers of the gastric wall with hypoechoic cancer tissue, but 5th (serosal) layer is not involved (T2 category).

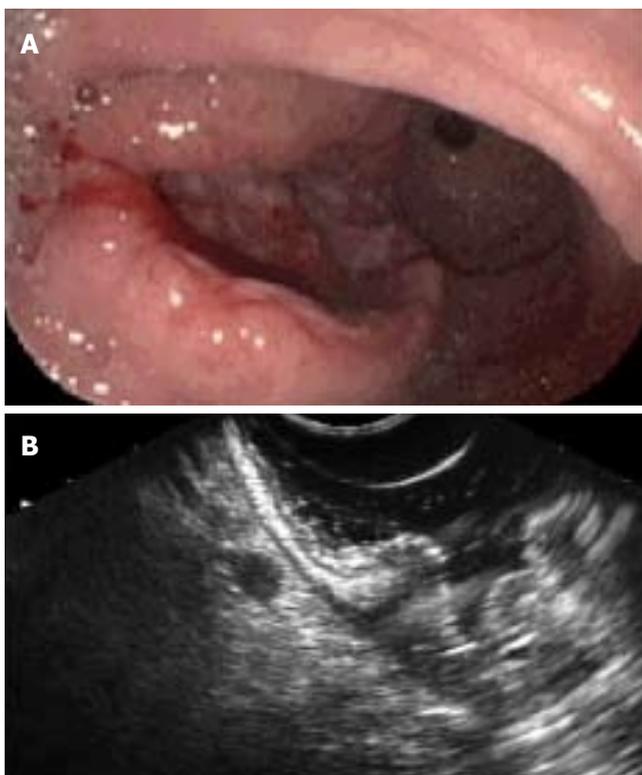


Figure 2 A case of advanced gastric cancer. **A:** Endoscopic view of Borrmann III type of gastric cancer; **B:** EUS image demonstrates T3 cancer with malignant lymph node. Note the hypoechoic structure and sharp margin of the lymph node (1.0 cm x 0.6 cm).

were found as surgically unresectable (T4 N+).

Pathohistologic findings

T staging: The diagnostic accuracy of EUS was 83.3% in T1 staging, 60.0% in T2 staging, 100% in T3 staging, and 25% in T4 staging, respectively. Twenty-eight of forty-one cancers were staged correctly and the overall diagnostic accuracy of T stage was 68.3%. Ten cases were overstaged

(24.4%) and 3 cases were understaged (7.3%) (Table 3). Echoendoscopic features of early and advanced gastric cancer are presented in (Figures 1A-1D).

N staging: EUS correctly determined 27 of 41 patients with the overall accuracy of 66.0%. The accuracy of EUS in N0 staging was high, all 17 patients without malignant lymph node metastasis were diagnosed correctly. However, EUS findings of preoperative positive metastatic lymph nodes in 10 patients were not confirmed histopathologically, and the accuracy of EUS in N+ staging was 41.7% (Table 4). The endosonographic features of advanced gastric cancer with malignant lymph nodes are shown in Figure 2.

DISCUSSION

The accurate staging of gastric cancer is the most important prognostic factor for patient management and EUS is the most reliable method in T and N staging of gastric cancer with high diagnostic rates. Such an accurate staging will apply the stage-dependent correct management of the patients (radical surgery or palliative treatment) and will provide a great benefit avoiding unnecessary laparotomy on patients with unresectable disease. EUS is considered as the most accurate modality for T staging of gastric cancer in comparison with CT and intraoperative assessment^[10,11].

The accuracy of EUS for gastric cancer from different authors ranges 64.8% - 92% in T staging and 50% - 90% in N staging (Table 5). These studies demonstrated that EUS is the most accurate staging method for gastric cancer with a few incidences of overstaging and understaging. The excellent results of accuracy of both T and N staging are shown in a study by Botet *et al.*^[12] to be 92% and 78%, respectively. The high accuracy of EUS in preoperative staging of gastric cancer is proved by our results. In the current study, EUS had a diagnostic accuracy of 68.3% for tumor invasion. EUS had 24.4% overstaging in T staging, 2 of the 12 T1 tumors overstaged as T2, 3 of the 20 T2 tumors overstaged as T3. The main reason of

Table 5 Literature summary of EUS studies on gastric cancer

| | Author | Period | Number of patients | Accuracy (%) | |
|----|---|-------------------|--------------------|--------------|--------------|
| | | | | T stage | N stage |
| 1 | Botet <i>et al.</i> ^[12] (USA) | 1986–1988 | 50 | 92 | 78 |
| 2 | Akahoshi <i>et al.</i> ^[13] (Japan) | 1986–1990 | 74 | 81.1 | 50 |
| 3 | Ziegler <i>et al.</i> ^[11] (Germany) | 1986–1990 | 108 | 86 | 74 |
| 4 | Lightdale ^[9] (USA) | 1989–1991 | 525 | 81 | 76 |
| 5 | Dittler <i>et al.</i> ^[14] (Germany) | 1989–1992 | 264 | 83 | 66 |
| 6 | Francois <i>et al.</i> ^[15] (France) | 1991–1993 | 35 | 79 | 79 |
| 7 | Yanai <i>et al.</i> ^[16] (Japan) | 1990–1995 | 104 | 64.8 | Early stage |
| 8 | Meining <i>et al.</i> ^[17] (Germany) | 1992–1996 | 33 | 66 | Not reported |
| 9 | Yanai <i>et al.</i> ^[18] (Japan) | 1996–1997 | 52 | 71 | Early stage |
| 10 | Guo <i>et al.</i> ^[19] (China) | 1996–1997 | 62 | 83.9 | 79 |
| 11 | Hunerbein <i>et al.</i> ^[20] (Germany) | 1997 ¹ | 30 | 82 | 80 |
| 12 | Habermann <i>et al.</i> ^[21] (Germany) | 1998–2000 | 51 | 86 | 90 |
| 13 | Hizawa <i>et al.</i> ^[22] (Japan) | 1997–2002 | 234 | 78 | Early stage |
| 14 | Xi <i>et al.</i> ^[23] (China) | 2002 ¹ | 32 | 80 | 68.6 |
| 15 | Shimoyama <i>et al.</i> ^[24] (Japan) | 1996–2003 | 45 | 71 | 80 |
| 16 | Bhandari <i>et al.</i> ^[10] (Korea) | 2003 | 63 | 87.5 | 79.1 |

¹Year of article publication/duration of study not reported.

overstaging in T1 cancer is the thickening of gastric wall due to perifocal inflammatory reaction, which is difficult to distinguish from cancer tissue and imitates the presence of T2 cancer. Absence of serosal layer in certain regions of the stomach, the lesser curvature, the posterior wall of fundus and the anterior wall of antrum is the reason for overstaging T2 cancer. Cancers of these areas are classified histopathologically as T2 cancer, even carcinoma infiltrates through the whole gastric wall, because no serosal infiltration can be found.

EUS accuracy of metastatic lymph node involvement was 66% in the present study. Such slightly lower accuracy is related to the absence of standard differential echoendoscopic criteria for benign and malignant lymph nodes. Echoendoscopic features of metastatic lymph nodes from different authors include size > 10 mm, rounded structure, sharp demarcation of borders, and hypoechoic (dark) structure^[25,26]. However, endoscopic ultrasonographic detection of metastatic lymph nodes is complicated, due to the difficulty of differentiation between malignant and inflammatory lymph nodes. Francois *et al.*^[15] described that hypoechoic lymph nodes with well-defined margins and largest diameter/smallest diameter ratio less than 2 are considered to be malignant. Dittler and Siewert^[14] noticed that, if EUS does not diagnose malignant lymph nodes in T1 or T2 stage, stage N0 can be assumed; if lymph nodes are visualized in stages T3 and T4, then they tend to be malignant. Results of certain studies^[27] demonstrated that the EUS-guided fine-needle aspiration biopsy would be very useful to distinguish between benign and malignant lymph nodes.

Other reasons for inaccuracy of evaluation of tumor lymph nodes are related to the limited depth of transducer, and unsatisfactory visualization of distant lymph node by EUS. EUS cannot permit the assessment of tissue beyond the depth of about 5–6 cm.

The presence of ascites in gastric cancer patients is a poor prognostic sign and implies the presence of peritoneal metastasis. EUS-guided fine-needle aspiration biopsy also has been successfully used to detect malignant ascites^[28–30]. EUS detection of distant metastatic lymph nodes and distant metastasis or M staging of gastric cancer

is insufficient due to limited penetration depth of this method as mentioned above. Therefore, combined use of EUS and CT, which is superior to EUS for gaining information about distant metastasis, should be effective for the management of gastric cancer patients for appropriate treatment options.

In conclusion, EUS is a useful diagnostic method for accurate preoperative staging for T and N criteria for gastric cancer. The accurate preoperative staging is extremely essential for proper stage-depending patient management, which improves the 5-year survival rate of this dismal prognostic disease. However, EUS evaluation of malignant lymph nodes is still unsatisfactory. Therefore, great effort should be taken to study differential criteria of malignant lymph nodes from benign lymph nodes.

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LIVER CANCER

Hepatocellular carcinoma in extremely elderly patients: An analysis of clinical characteristics, prognosis and patient survival

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positive rate for HBsAg was significantly lower in the extremely elderly group and the proportion of patients negative for HBsAg and HCVAb obviously increased in the extremely elderly group ($P < 0.001$). There were no significant differences in the following parameters: diameter and number of tumors, Child-Pugh grading, tumor staging, presence of portal thrombosis or ascites, and positive rate for HCVAb. Extremely elderly patients did not often receive surgical treatment ($P < 0.001$) and they were more likely to receive conservative treatment ($P < 0.01$). There were no significant differences in survival curves based on the Kaplan-Meier methods in comparison with the overall patients between the two groups. However, the survival curves were significantly worse in the extremely elderly patients with stage I/II, stage I/II and Child-Pugh grade A cirrhosis in comparison with the non-elderly group. The causes of death did not differ among the patients, and most cases died of liver-related diseases even in the extremely elderly patients.

CONCLUSION: In the patients with good liver functions and good performance status, aggressive treatment for HCC might improve the survival rate, even in extremely elderly patients.

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Key words: Hepatocellular carcinoma; Extremely elderly patients; Survival analysis; Cause of death

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<http://www.wjgnet.com/1007-9327/12/48.asp>

Abstract

AIM: To identify the clinical and prognostic features of patients with hepatocellular carcinoma (HCC) aged 80 years or more.

METHODS: A total of 1310 patients with HCC were included in this study. Ninety-one patients aged 80 years or more at the time of diagnosis of HCC were defined as the extremely elderly group. Two hundred and thirty-four patients aged ≥ 50 years but less than 60 years were regarded as the non-elderly group.

RESULTS: The sex ratio (male to female) was significantly lower in the extremely elderly group (0.90:1) than in the non-elderly group (3.9:1, $P < 0.001$). The

INTRODUCTION

With the arrival of the aging society, an increasing number of elderly patients with cancer is predicted in the future. Hepatocellular carcinoma (HCC) is one of the

Table 1 Age distribution of the 1 310 patients with hepatocellular carcinoma

| Age (yr) | Number of HCC patients |
|----------|------------------------|
| <30 | 3 |
| 30-40 | 4 |
| 40-50 | 41 |
| 50-60 | 234 |
| 60-70 | 485 |
| 70-80 | 452 |
| 80< | 91 |
| Total | 1 310 |

most common cancers and the age distribution of HCC patients has steadily increased because of the improved management of chronic liver diseases^[1-5]. As a result, we sometimes encounter patients with HCC aged 80 years or more. However, the clinical characteristics and the long-term prognosis of these patients still remain obscure because they often have concomitant diseases and therefore long-term follow-up for such patients remains difficult. In recent studies, “the elderly” have usually been defined as to be at the ages of 60, 65, or 70 years and above^[1-4]. The average life expectancy of Japanese males is 78.36 years, while that of females is 85.33 years^[6]. With the increase in the average lifetime, the age at which a person is considered elderly is rising. Clarifying the optimal treatment strategy for extremely elderly patients with HCC has thus become an urgent necessity. However, to our knowledge, there have been so far few reports evaluating extremely elderly patients with HCC aged 80 years or more^[5]. Hence, in this study, we aimed to clarify the age-specific clinical characteristics of HCC, and to evaluate the survival and characteristics of extremely elderly patients. We, therefore, undertook a retrospective study of 91 extremely elderly patients with HCC aged 80 years or more in comparison to the non-elderly patients from 1 310 consecutive HCC patients.

MATERIALS AND METHODS

A total of 1 310 consecutive patients were enrolled in this study. The patients were newly diagnosed with HCC and observed from January 1992 to December 2003 at the Department of Medicine and Molecular Science, Gunma University Graduate School of Medicine and nine affiliated hospitals, namely, Maebashi Red Cross Hospital, Iseaki Municipal Hospital, Kiryu Kousei General Hospital, Tone Chuo Hospital, National Nishigunma Hospital, Saiseikai Maebashi Hospital, Public Tomioka General Hospital, Fuji Heavy Industries Ltd, Health Insurance Society General Ota Hospital, and Shimada Memorial Hospital. A diagnosis of HCC was confirmed histopathologically or clinicopathologically from biopsy specimens or combined examinations of ultrasonography, computed tomography, and selective angiography. For each patient, the following data were recorded: age, sex, hepatitis B surface antigen (HBsAg), hepatitis C virus antibody (HCVAb), biochemical analysis (total bilirubin, albumin, prothrombin time, platelet count, and ICG R15), serum alpha-fetoprotein (AFP), protein induced by vitamin K absence

or antagonist II (PIVKA-II), diameter and number of tumors, Child-Pugh grading, tumor staging, presence of portal thrombosis or ascites, initial therapy, and survival. The AFP level was divided into two categories: 20 µg/L or less, and more than 20 µg/L. PIVKA-II level was also divided into two categories: < 40 and ≥ 40 AU/L. The diameter of the largest tumor was measured in its greatest dimension if the patient had two or more tumors. The number of HCCs was divided into two groups: solitary and non-solitary tumors. Portal thrombosis was defined as a protrusion of the tumor into the first and/or second branch, or into the main trunk of the portal vein. The presence of concomitant disease with a strong impact on the prognosis (e.g., malignant neoplasm, cardiovascular disease, and cerebrovascular disease) was recorded. The types of initial treatment for HCC were categorized into five categories: (1) transcatheter arterial embolization (TAE) or transcatheter arterial infusion (TAI); (2) percutaneous injection or ablation [percutaneous ethanol injection (PEI), microwave ablation (MA), or radiofrequency ablation (RFA)]; (3) surgical resection, including liver transplantation; (4) systemic or reservoir chemotherapy; and (5) supportive care. According to their age at the initial diagnosis, the patients were categorized into two groups: an extremely elderly group consisting of 91 HCC patients aged ≥ 80 years and a non-elderly group comprising 234 HCC patients aged ≥ 50 years but < 60 years.

Statistical analysis

Differences in the proportions were evaluated by Fisher's exact probability test. Differences in the means were evaluated by the Student's *t*-test. The survival curves according to the Kaplan-Meier method were compared using the log-rank test. Using Cox's proportional hazard model, a multivariate analysis was performed to evaluate the prognostic factors. *P* < 0.05 was considered statistically significant.

RESULTS

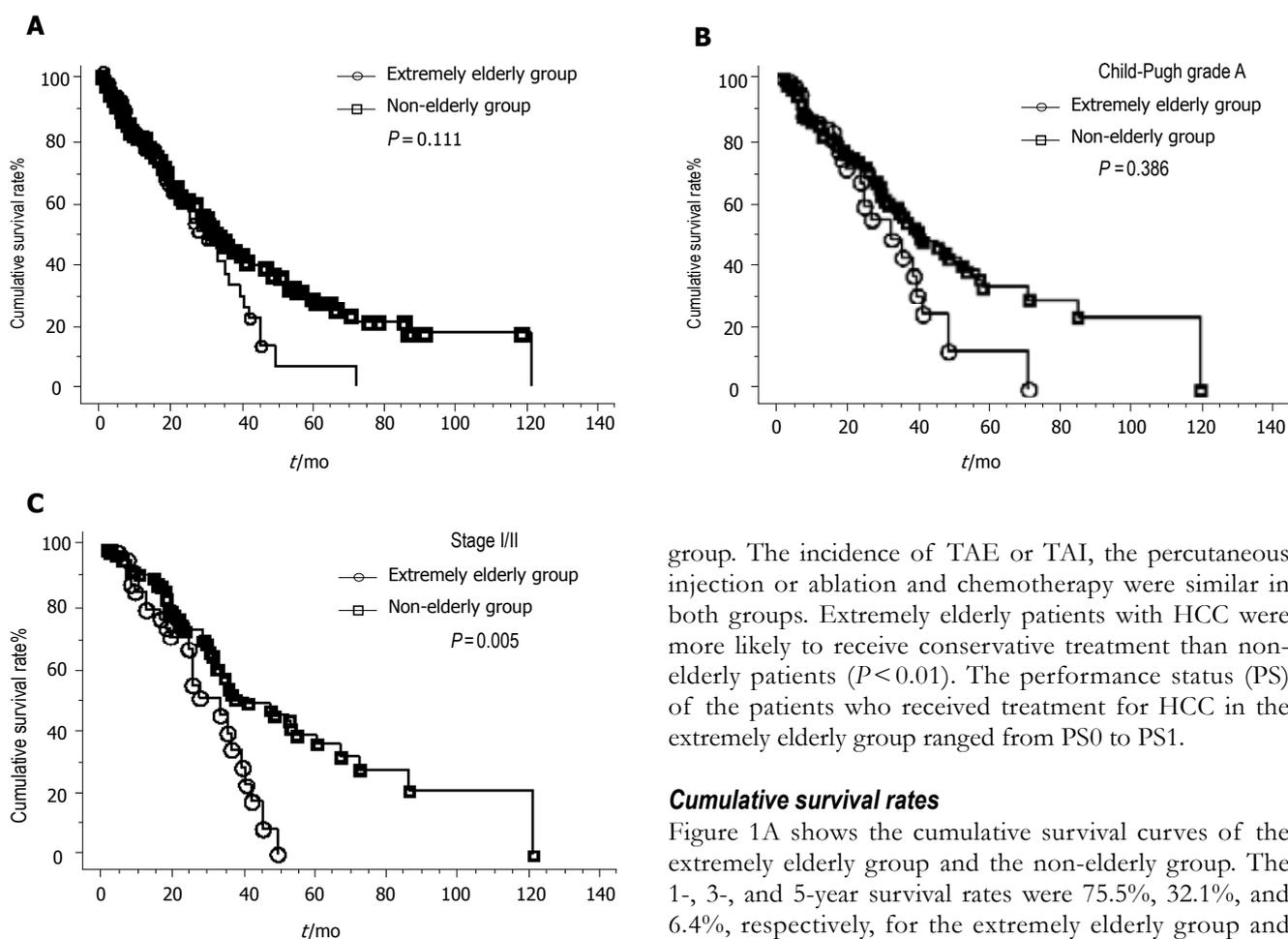
Characteristics of extremely elderly and non-elderly groups

The age distribution of 1 310 patients with HCC is shown in Table 1. The mean age of the 1 310 patients with HCC was 66.7 ± 9.3 years (range, 20-94 years; median, 68 years). There were 91 patients who were older than 80 years, and 234 patients aged ≥ 50 years but less than 60 years. The characteristics of the extremely elderly group and the non-elderly group are summarized in Table 2. The sex ratio, with a comparable male to female ratio was 0.90:1 in the extremely elderly group and 3.9:1 in the non-elderly group, showing that women were more prevalent in the extremely elderly group (*P* < 0.001). The positive rate for HBsAg was significantly lower in the extremely elderly group (*P* < 0.001). The proportion of the patients negative for HBsAg and HCVAb markedly increased in the extremely elderly group (*P* < 0.001). The prothrombin time and platelet count were significantly higher in the extremely elderly group (*P* < 0.001 and *P* < 0.01, respectively), while ICG R15 was significantly lower in the extremely elderly group (*P* < 0.001) than in the non-elderly group.

Table 2 Profile of 91 extremely elderly patients and 34 non-elderly patients with hepatocellular carcinoma

| | Extremely elderly (80 yr or more) | Non-elderly (50-60 yr old) | P-value |
|-------------------------------|--------------------------------------|-------------------------------|-------------|
| Sex (M/F) | 43/48 | 186/48 | $P < 0.001$ |
| Mean age (range) | 82.3 ± 2.7 (80-94) | 55.3 ± 2.9 (50-59) | $P < 0.001$ |
| HBsAg (+/-) | 3/88 | 36/198 | $P < 0.001$ |
| HCV (+/-) | 67/24 | 182/52 | NS |
| non B, nonC/B or C | 21/70 | 16/218 | $P < 0.001$ |
| Diameter of HCC (mm) | 39.5 ± 24.3 | 36.8 ± 27.0 | NS |
| Number of HCC (1/2 or more) | 47/44 | 110/124 | NS |
| Child-Pugh grading (A/B/C/uk) | 62/22/3/4 | 144/67/18/6 | NS |
| Stage (I/II/III/IVA/IVB/uk) | 11/37/25/11/3/4 | 44/85/53/36/10/5 | NS |
| Portal thrombus (-/+) | 77/14 | 189/45 | NS |
| Ascites (-/+) | 69/22 | 191/43 | NS |
| T-Bil (μmol/L) | 1.23 ± 1.29 | 1.5 ± 1.82 | NS |
| Alb (g/dL) | 3.55 ± 0.53 | 3.58 ± 0.6 | NS |
| PT (%) | 83.3 ± 15.9 | 73.6 ± 18.4 | $P < 0.001$ |
| Plt (x 10 ⁴ /μL) | 13.5 ± 7.7 | 10.7 ± 5.8 | $P < 0.01$ |
| ICGR15 (%) | 22.6 ± 11.8 | 31.5 ± 18.7 | $P < 0.001$ |
| AFP (-/+/uk) | 33/53/5 | 75/150/9 | NS |
| PIVKA II (-/+/uk) | 35/51/5 | 116/109/9 | NS |

uk; unknown

**Figure 1** Survival curves of extremely elderly group and non-elderly group. **A:** All patients; **B:** Child-Pugh grade A patients; **C:** stage I/II patients.

There were no statistical differences in the prevalence of HCVAb, total bilirubin, albumin, AFP, PIVKAI, diameter and number of tumors, Child-Pugh grading, tumor stage, and presence of portal thrombosis or ascites. Table 3 shows the type of initial treatment. None of the patients underwent a surgical resection in the extremely elderly

group. The incidence of TAE or TAI, the percutaneous injection or ablation and chemotherapy were similar in both groups. Extremely elderly patients with HCC were more likely to receive conservative treatment than non-elderly patients ($P < 0.01$). The performance status (PS) of the patients who received treatment for HCC in the extremely elderly group ranged from PS0 to PS1.

Cumulative survival rates

Figure 1A shows the cumulative survival curves of the extremely elderly group and the non-elderly group. The 1-, 3-, and 5-year survival rates were 75.5%, 32.1%, and 6.4%, respectively, for the extremely elderly group and 79.3%, 43.8%, and 26.5%, respectively, for the non-elderly group. The difference in the survival rates between the two groups was not statistically significant ($P = 0.114$). Figure 1B shows the cumulative survival curves of the patients with Child-Pugh grade A in the two groups. Although the survival tended to be worse in the extremely elderly patients, it did not reach statistical significance ($P = 0.111$). There were also no significant differences in survival curves regarding the Child-Pugh grade B/C patients ($P = 0.386$). Due to the small number of patients with stage I tumors in the extremely elderly group, we compared

Table 3 Types of initial treatment for patients with hepatocellular carcinoma.

| Treatment | Extremely elderly (80 yr or more) | Non-elderly (50-60 yr old) | P-value |
|---------------------------------|-----------------------------------|----------------------------|-------------|
| TAE or TAI | 59 | 128 | NS |
| PEI or RFA or MA | 18 | 62 | NS |
| Surgery (liver transplantation) | 0 | 28 (3) | $P < 0.001$ |
| Chemotherapy | 2 | 7 | NS |
| Supportive care | 12 | 9 | $P < 0.01$ |

TAE, Transcatheter arterial embolization; TAI, Intraarterial infusion; PEI, percutaneous ethanol injection; MA, Microwave ablation; RFA, Radiofrequency ablation; NS, not significant.

Table 4 Causes of death in extremely elderly patients with HCC

| Cause of death | Extremely elderly (80 yr or more) | Non-elderly (50-60 yr old) | P-value |
|---------------------------|-----------------------------------|----------------------------|---------|
| HCC | 27 | 77 | NS |
| Hepatic failure | 17 | 25 | |
| Gastrointestinal-Bleeding | 4 | 6 | |
| Others | 7 | 9 | |
| Unknown | 0 | 6 | |

the patients with stage I/II tumors between both groups. Figure 1C shows the cumulative survival curves of the stage I/II patients in both groups. The survival curves were significantly worse in the extremely elderly patients with stage I tumors in the extremely elderly group, we compared the patients with stage I/II tumors between both groups. Figure 1C shows the cumulative survival curves of the stage I/II patients for both groups. The survival curves were significantly worse in the extremely elderly patients with stage I/II tumors ($P = 0.005$). However, there were no significant differences in survival curves regarding stage III/IV ($P = 0.479$) or stage IV ($P = 0.794$) disease. In stage I/II and Child-Pugh grade A patients, the survival curves were significantly worse in the extremely elderly patients ($P = 0.005$). Survivals adjusted for the etiology of liver disease were also calculated. No significant differences were observed in the survival curves regarding HCV-related disease between the extremely elderly and non-elderly groups ($P = 0.142$). There were also no significant differences in the survival curves of the patients negative for HBsAg and HCVAb between the two groups ($P = 0.447$). Due to the small number of patients positive for HBsAg in the extremely elderly group, we could not compare those patients between both groups.

To evaluate the prognostic factors for the extremely elderly HCC patients and non-elderly patients, we performed a multivariate analysis using Cox's proportional hazard model, which showed that the Child-Pugh grading ($P < 0.05$), tumor staging ($P < 0.01$), albumin ($P < 0.01$), and platelet count ($P < 0.05$) were prognostic factors for extremely elderly patients with HCC. The Child-Pugh grading ($P < 0.05$), tumor staging ($P < 0.05$), and diameter of tumors ($P < 0.01$) were found to be prognostic factors for the non-elderly patients with HCC. The age, sex, HBsAg, HCVAb, total bilirubin, prothrombin time,

number of tumors, and presence of portal thrombosis or ascites were not found to be prognostic factors, based on Cox's proportional hazard model. In the overall patients with HCC, the Child-Pugh grading ($P < 0.05$), tumor staging ($P < 0.05$) and diameter of tumors ($P < 0.01$) were found to be prognostic factors. The age was not found to be associated with the prognosis of the overall patients with HCC.

Cause of death of extremely elderly patients with HCC

Of the 91 patients aged ≥ 80 years, 55 (60.4%) patients died during the observation period table 4. The causes of death among the 55 patients were HCC-related or hepatic failure in 44 patients (80.0%), gastrointestinal bleeding, including the rupture of esophageal varices in 4 patients (7.3%), and other diseases not related to HCC or liver cirrhosis in 7 patients (12.7%), such as pneumonia, renal failure and brain bleeding. In the non-elderly group, 123 (52.6%) patients died during the observation period. The causes of death among the 123 patients were HCC-related or hepatic failure in 102 patients (82.9%), gastrointestinal bleeding, including rupture of esophageal varices in six patients (5.0%), and other diseases not related to HCC or liver cirrhosis in nine (7.3%) and no records in six patients (5.0%).

Concomitant diseases of the patients with HCC

Of the 91 patients, 71 (78.0%) patients had concomitant diseases with liver disease in the extremely elderly group. As shown in Table 5, a variety of concomitant diseases were observed in the extremely elderly patients. "Others" in malignant neoplasms included: one ureteral carcinoma, one skin cancer, and one malignant lymphoma. "Other diseases" included: three benign prostatic hypertrophy, one idiopathic thrombocytopenia, one lichen planus, one ovarian cyst, one adrenal adenoma, and six others. On the other hand, 89 of 234 patients (38.0%) had concomitant diseases with liver disease in the non-elderly group. The ratio of patients having concomitant diseases was significantly higher in the extremely elderly group ($P < 0.001$).

DISCUSSION

Although the survival of the patients with an early stage and a good liver function was better in the non-elderly group, we could not find any significant difference in overall survival curves or causes of death between the extremely elderly and non-elderly groups with HCC. Even though the extremely elderly patients had various concomitant diseases, most of the patients demonstrating HCC died from HCC-related causes. As a result, HCC was a life-limiting factor even though the patients were extremely elderly. In Japan, based on data from 2003^[6], the average life expectancies at birth are 78.36 years for males and 85.33 years for females. In addition, an 80-year-old male has an average life expectancy of 8.26 years, while a female aged 80 years can expect to live another 11.04 years^[6]. The 5-year survival rate in the extremely elderly group with HCC was only 6.4% in this study. We, therefore, have to treat HCC even though such patients are over 80 years of age and have concomitant diseases.

Table 5 Concomitant diseases in extremely elderly patients with hepatocellular carcinoma.

| Concomitant disease | | Extremely elderly (80 yr or more) | Non-elderly (50-60 yr old) |
|----------------------------------|------------------------------------|-----------------------------------|----------------------------|
| Malignant neoplasm | Gastric cancer | 2 | 2 |
| | Colon cancer | 3 | 4 |
| | Prostatic cancer | 2 | 0 |
| | Others | 3 | 1 |
| Cardiovascular disease | Ischemic heart disease | 9 | 3 |
| | Hypertension | 36 | 32 |
| | Arrhythmia | 7 | 2 |
| | Congestive heart failure | 2 | 0 |
| | Valvular heart disease | 2 | 0 |
| | Aortic aneurysms | 2 | 0 |
| Respiratory disease | Chronic bronchitis | 3 | 0 |
| | Interstitial pneumonia | 2 | 1 |
| | Pneumonia | 2 | 0 |
| | Others | 3 | 1 |
| Gastrointestinal disease | Gall stone | 4 | 3 |
| | Chronic pancreatitis | 1 | 4 |
| | Peptic ulcer | 2 | 5 |
| | Reflux esophagitis | 3 | 1 |
| Endocrinal disease | Diabetes mellitus | 23 | 47 |
| | Gout | 2 | 6 |
| | Others | 0 | 2 |
| Neurological and cerebrovascular | Cerebral infarction or hemorrhage | 7 | 2 |
| | Dementia | 3 | 0 |
| | Others | 0 | 2 |
| Renal disease | Chronic renal failure or nephritis | 3 | 2 |
| Other disease | | 13 | 5 |
| No concomitant disease | | 20 | 145 |

Dohmen *et al.*^[5] analyzed 36 patients aged ≥ 80 years and concluded that the survival rates are not significantly different from the non-elderly group, and that the advanced stage of HCC, not an advanced age, has the greatest influence on the survival rate in extremely elderly patients^[5]. Hoshida *et al.*^[8] evaluated 135 patients with chronic liver disease aged ≥ 80 years and found that most patients (63.5%) would die of diseases other than liver diseases, such as pneumonia, especially in the non-cirrhosis group. It is reasonable that the extremely elderly patients without HCC die of diseases other than liver diseases. However, in their study, 18 patients with HCC at the start of observations had a poor prognosis.

Although there was no difference in the Child-Pugh grading, the extremely elderly group had good liver function as indicated by the prothrombin time or ICGR15 level. We speculated that patients with a poor liver function might die of liver failure and will not be able to live until 80 years of age when complicated with HCC. Otherwise, not only the liver function, but also other genetic or environmental factors may contribute to the development of HCC in extremely elderly patients. In this study, extremely elderly patients with HCC had a relatively good PS. The 10 hospitals included in this study

are core hospitals for each area. The patients with a poor PS or poor liver function might have been excluded by the primary physician for aggressive treatment of HCC and thus might not have been introduced to these 10 hospitals.

Females were more prevalent in the extremely elderly group ($P < 0.001$). The proportion of females in the general population is known to gradually increase with increasing age^[6]. Males are usually dominant in non-elderly patients with HCC and their prognosis of HCC is quite poor. We speculated that males with HCC might die of liver-related diseases before 80 years of age. However, we could not determine the reason why the female ratio significantly increased in the extremely elderly group of HCC. Previous studies reported that HBV-associated HCC is common in younger patients in Japan^[11-13]. Our results were compatible with our expectation because only 3.3% of the HBV-associated HCC was identified in the extremely elderly group in contrast to 15.3% in the non-elderly group ($P < 0.001$). The ratio of non B and non C hepatitis patients significantly increased in the extremely elderly group. Other factors except for hepatitis virus infection such as alcohol or genetic disturbance may thus contribute to the development of HCC in the extremely elderly group.

Regarding the initial treatment, Collier *et al.*^[10] reported in 1994 that elderly patients (≥ 65 years) with HCC are more likely to receive conservative treatment than younger patients (< 65 years), despite a similar disease stage. Conversely, Poon *et al.*^[3] concluded that a hepatic resection and TAE for HCC in elderly patients (≥ 70 years) are well tolerated and show an improved survival rate. Percutaneous localized therapies or TAE were similarly performed in both the extremely elderly and non-elderly groups in this study and there were no obvious differences regarding adverse effects. However, no surgical resections were performed in the extremely elderly group. The oldest patient receiving a surgical resection as the initial treatment among these 1 310 patients was 77 years. The extremely elderly patients with HCC were more likely to receive conservative treatment than the non-elderly patients. Non-invasive treatments may have been selected due to the patient age or quality of life. Indeed, post-operative complications, such as pneumonia, show an incremental risk with age^[14]. The elderly patients are more liable to succumb to post-operative organ failure and complications, especially infections^[14]. The PS and physiological age are therefore considered to be more important than the chronological age in elderly patients with HCC.

In conclusion, there is no statistical difference in the overall survival rate between the extremely elderly and non-elderly groups after the diagnosis of HCC. HCC is considered to be a life-limiting factor even in extremely elderly patients. Therefore, in patients with good liver functions and good PS, aggressive treatment of HCC might improve the survival rate, even in extremely elderly patients.

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COLORECTAL CANCER

Clinical significance of subcellular localization of KL-6 mucin in primary colorectal adenocarcinoma and metastatic tissues

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cytoplasm was significantly associated with the presence of venous invasion ($P = 0.0003$), lymphatic invasion ($P < 0.0001$), lymph node metastasis ($P < 0.0001$), liver metastasis ($P = 0.058$), and advanced histological stage ($P < 0.0001$). Positive staining was observed in all metastatic lesions tested as well as in the primary colorectal carcinoma tissues.

CONCLUSION: The subcellular staining pattern of KL-6 in colorectal adenocarcinoma may be an important indicator for unfavorable behaviors such as lymph node and liver metastasis, as well as for the prognosis of patients.

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Key words: KL-6 mucin; Colorectal carcinoma; Metastasis; Prognosis; Immunohistochemistry

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Abstract

AIM: To assess subcellular localization of KL-6 mucin and its clinicopathological significance in colorectal carcinoma as well as metastatic lymph node and liver tissues.

METHODS: Colorectal carcinoma tissues as well as metastatic lymph node and liver tissues were collected from 82 patients who underwent colectomy or hepatectomy. Tissues were subjected to immunohistochemical analysis using KL-6 antibody.

RESULTS: Of the 82 colorectal carcinoma patients, 6 showed no staining, 29 showed positive staining only in the apical membrane, and 47 showed positive staining in the circumferential membrane and/or cytoplasm. Positive staining was not observed in non-cancerous colorectal epithelial cells surrounding the tumor tissues. The five-year survival rate was significantly lower in cases showing positive staining in the circumferential membrane and/or cytoplasm (63.0%) than those showing positive staining only in the apical membrane (85.7%) and those showing no staining (100%). Statistical analysis between clinicopathological factors and subcellular localization of KL-6 mucin showed that KL-6 localization in the circumferential membrane and/or

INTRODUCTION

MUC1, a transmembrane glycoprotein^[1, 2], has been detected in various cancer cell lines and secretory epithelial cells lining the respiratory, reproductive, and gastrointestinal tracts^[3-6]. It has been suggested that MUC1 may influence cell-to-cell adhesion, diminish the immune response, and be involved in intracellular signaling^[7, 8]. In carcinoma cells, it has been reported that high levels of MUC1 expression correlate with the invasive characteristic of tumors^[9-13]. Our latest study has also shown that aberrant expression of MUC1, which was detected by KL-6 antibody, is associated with cancer progression in the carcinoma of the ampulla of Vater^[14].

In normal epithelium, MUC1 is predominantly present on the apical surface of the epithelial cells^[15, 16]. Recently, it has been reported that, in breast carcinoma, MUC1 is expressed not only on the apical surface but also on

circumferential and basal membranes and in the cytoplasm of the carcinoma cells^[15,16]. Furthermore, this aberrant localization of MUC1 has been reported to be associated with worse prognosis for the patient^[17]. These findings suggest that subcellular observation of MUC1 expression in carcinoma cells is likely important for understanding the function of MUC1 and improving the prediction of prognosis. However, little is known about the clinical significance of subcellular localization of KL-6 mucin in other carcinomas.

In this study, we focused on subcellular localization of MUC1 in colorectal carcinoma as well as in the metastatic lymph nodes and liver tissue. MUC1 expression was immunohistochemically detected using KL-6 antibody, which recognizes the sialylated oligosaccharide moiety of MUC1 as a part of an epitope^[18]. In colorectal carcinoma tissues, it has been suggested that the expression of KL-6 mucin is associated with tumor aggressiveness^[10,19]. However, the subcellular localization and physiological function of KL-6 mucin in colorectal carcinoma have remained unknown. In this paper, we report that aberrant subcellular expression of KL-6 mucin in the circumferential membrane and/or cytoplasm is associated with lymph node and liver metastases and worse prognosis in colorectal carcinoma.

MATERIALS AND METHODS

Patients

Colorectal carcinoma tissues were collected from 82 consecutive patients (55 males and 27 females; 64.5 ± 11.4 years, mean \pm SD) with a single primary colorectal adenocarcinoma who underwent surgical resection at the Department of Surgery, Graduate School of Medicine, the University of Tokyo, between January 1991 and December 1992. For all cases with lymph node and liver metastasis, whole specimens of resected lymph nodes and metastatic liver tissues were collected from 36 and 7 patients, respectively, in the study group. All specimens were classified according to Japanese Classification of Colorectal Carcinoma by the Japanese Society for Cancer of the Colon and Rectum^[20], including the status of lymph node and liver metastasis at the time of surgical intervention and the depth of invasion (m, invasion of mucosa; sm, invasion of submucosa; mp, invasion of muscularis propria; ss, invasion of subserosa or subadventitia; se, invasion of serosa or adventitia; and si, invasion of adjacent structures).

Immunohistochemical staining

The immunohistochemical staining approach matched that of previous studies^[14]. Briefly, 4 μ m-thick sections were cut from archival formalin-fixed paraffin-embedded tissue blocks, deparaffinized, and dehydrated using a graded series of ethanol solutions. Endogenous peroxidase activity was halted through administration of 3 mL/L hydrogen peroxide/methanol for 30 min. The slides were rinsed with phosphate-buffered saline and then blocked with normal goat serum for 30 min at room temperature. The sections were then incubated with a KL-6 monoclonal antibody solution (1:200 dilution; Eisai, Tokyo, Japan)

for 60 min at room temperature. After the sections were incubated with biotinylated secondary antibody for 60 min, bound biotinylated antibody was then tested by the biotin-streptavidin-peroxidase complex method following the manufacturer's instructions (Histofine SAB-PO kit; Nichirei, Tokyo, Japan). 3,3'-Diaminobenzidine was used as the chromogen, and hematoxylin was used as a counterstain. The negative control sections were treated by omitting the primary antibody to monitor background staining.

Evaluation of immunohistochemically stained carcinomas

Overall staining was evaluated in carcinoma cells observed in 10 random microscopic fields, or in the entire area if the tissue sample comprised less than 10 fields. Subcellular staining patterns were recorded by judging the apical membrane, circumferential membrane, and cytoplasm as described elsewhere^[17]. Three investigators (Q.G., W.T., and N.K.) separately judged the staining characteristics, and the discrepancies were resolved through mutual observation and discussion of the microscopic fields.

Statistical analysis

The χ^2 -test was used to evaluate the relationship between staining pattern and clinicopathological parameters. Survival curves were calculated using the Kaplan-Meier method and compared with the results of the log-rank test. Two patients (one in the no-staining group, another in the apical membrane staining group) were excluded from the data analysis for survival because the cause of death for these patients was not colorectal cancer. $P < 0.05$ was considered statistically significant. Statview 5.0J (Abacus Concepts, Berkeley, CA, USA) statistical software was used for data analyses.

RESULTS

Subcellular localization of KL-6 mucin

Among the 82 cases of colorectal carcinoma, 76 cases showed positive staining of KL-6 mucin. As shown in Figure 1, there was a considerable heterogeneity in the subcellular localization of KL-6 mucin. Staining was observed in either the apical or circumferential membrane (Figures 1A and 1B). Some cases showed positive staining in the cytoplasm in addition to the membranous region (Figures 1C and 1D). The number of cases showing the respective subcellular staining patterns are summarized in Table 1. It is notable that cytoplasmic staining tended to be accompanied by positive staining in the circumferential membrane (37/45, 82%) rather than in the apical membrane (8/45, 18%). Positive staining was not observed in non-cancerous colorectal epithelial cells in any case of this study (data not shown).

Relationship between survival and subcellular localization of KL-6 mucin

The five-year survival rate was 85.7% for cases showing positive staining only in the apical membrane ($n = 28$), 61.5% for cases showing positive staining in the circumferential membrane ($n = 39$), and 64.4% for cases showing positive staining in cytoplasm ($n = 45$) (data

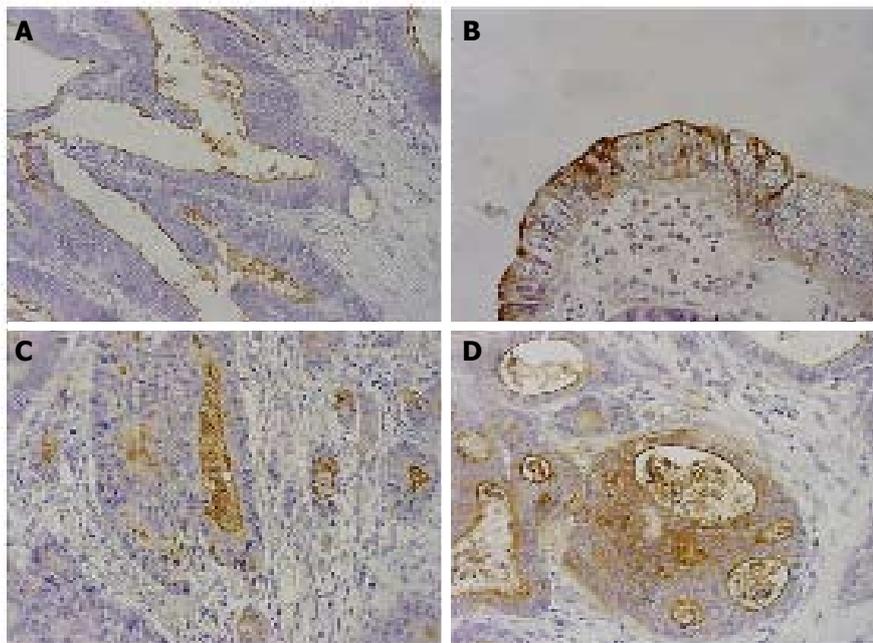


Figure 1 Immunohistochemical staining of colorectal adenocarcinoma tissues using KL-6 antibody ($\times 200$). **A**: Positive staining in the apical membrane; **B**: positive staining in the circumferential membrane; **C**: positive staining in the apical membrane and cytoplasm; **D**: positive staining in the circumferential membrane and cytoplasm.

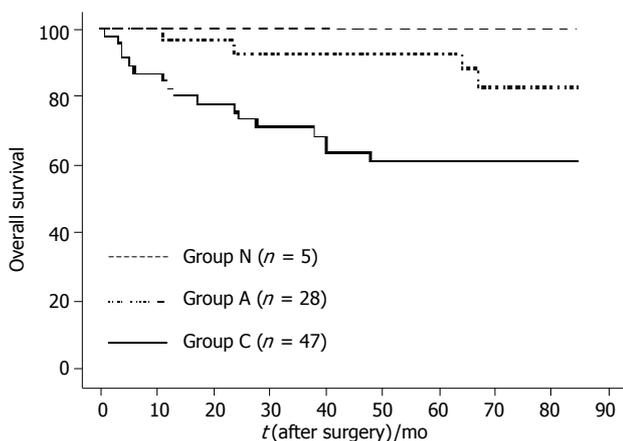


Figure 2 Kaplan-Meier curves for overall survival rates of patients with colorectal adenocarcinoma. Patients with KL-6 expression in the circumferential membrane and/or cytoplasm (solid line, group C, $n = 46$), in the apical membrane (dashed line, group A, $n = 29$) and without KL-6 staining (dotted line, group N, $n = 5$) were followed up for more than 70 mo. Two of 82 patients were excluded from the data analysis as described in Materials and Methods.

not shown). There were significant differences between the cases showing positive staining only in the apical membrane and the cases showing positive staining in the circumferential membrane ($P = 0.021$), and between the cases showing positive staining only in the apical membrane and the cases showing positive staining in cytoplasm ($P = 0.033$). On the other hand, the five-year survival rate was 100% for the cases showing no staining ($n = 5$). These results suggested that a subcellular KL-6 expression profile was associated with survival, and that cases showing positive staining in the circumferential membrane and/or cytoplasm showed worse prognosis.

As described above, cytoplasmic staining tended to be accompanied with positive staining of the circumferential membrane. Therefore, we classified the cases into the following three groups according to their subcellular staining profile: group N, negative ($n = 6$); group A,

Table 1 Summary of subcellular staining of KL-6 mucin in colorectal adenocarcinoma

| Group ¹ | KL-6 mucin staining | | | n |
|--------------------|---------------------|--------------------------|-----------|----|
| | Apical membrane | Circumferential membrane | Cytoplasm | |
| N | Negative | Negative | Negative | 6 |
| A | Positive | Negative | Negative | 29 |
| C | Positive | Negative | Positive | 8 |
| C | Negative | Positive | Negative | 2 |
| C | Negative | Positive | Positive | 37 |

¹Patient groups N, A, and C were categorized according to the subcellular expression profile of KL-6 mucin (see text).

positive only in the apical membrane ($n = 29$); and group C, positive in the circumferential membrane and/or cytoplasm ($n = 47$) (Table 1). As shown in Figure 2, the five-year survival rate was significantly lower in group C (63.8%) than that in group A (85.7%; $P = 0.029$). On the other hand, group N showed the highest five-year survival rate (100%).

Relationship between clinicopathological factors and subcellular localization of KL-6 mucin

The relationship between clinicopathological factors and subcellular KL-6 mucin staining of the colorectal adenocarcinomas is summarized in Table 2. Positive staining in the circumferential membrane and/or cytoplasm was significantly associated with the presence of venous invasion, lymphatic invasion, and lymph node metastasis. This subcellular staining characteristic was also associated with the progression of the depth of invasion and histological stage (Table 2). Notably, all cases having lymph node ($n = 36$) or liver metastasis ($n = 7$) showed positive staining in the circumferential membrane and/or cytoplasm. This suggested that aberrant subcellular expression of KL-6 mucin in the circumferential

Table 2 Relationship between clinicopathological factors and subcellular staining profile of KL-6 mucin in colorectal adenocarcinoma *n*(%)

| Factors | <i>n</i> | Subcellular staining profile of KL-6 mucin | | | <i>P</i> |
|------------------------------|----------|--|-----------------|---|----------|
| | | No staining | Apical membrane | Circumferential membrane and/or cytoplasm | |
| Age (yr) | | | | | 0.449 |
| ≤60 | 29 | 2 (6.9) | 12 (41.4) | 15 (51.7) | |
| >60 | 53 | 4 (7.5) | 17 (32.1) | 32 (60.4) | |
| Sex | | | | | 0.469 |
| Male | 55 | 4 (7.3) | 21 (38.2) | 30 (54.5) | |
| Female | 27 | 2 (7.4) | 8 (29.6) | 17 (63.0) | |
| Differentiation | | | | | 0.055 |
| Well | 50 | 6 (12.0) | 21 (42.0) | 23 (46.0) | |
| Moderate | 29 | 0 (0) | 8 (27.6) | 21 (72.4) | |
| Poor | 3 | 0 (0) | 0 (0) | 3 (100) | |
| Venous invasion | | | | | 0.0003 |
| (+) | 40 | 0 (0) | 9 (22.5) | 31 (77.5) | |
| (-) | 42 | 6 (14.3) | 20 (47.6) | 16 (38.1) | |
| Lymphatic invasion | | | | | <0.0001 |
| (+) | 24 | 0 (0) | 2 (8.3) | 22 (91.7) | |
| (-) | 58 | 6 (10.3) | 27 (46.6) | 25 (43.1) | |
| Depth of invasion | | | | | 0.009 |
| m | 3 | 3 (100) | 0 (0) | 0 (0) | |
| sm, mp | 14 | 2 (14.3) | 8 (57.1) | 4 (28.6) | |
| ss, se | 59 | 1 (1.7) | 20 (33.9) | 38 (64.4) | |
| si | 6 | 0 (0) | 1 (16.7) | 5 (83.3) | |
| Histological stage | | | | | <0.0001 |
| 0 | 3 | 3 (100) | 0 (0) | 0 (0) | |
| I | 12 | 2 (16.7) | 8 (66.6) | 2 (16.7) | |
| II | 29 | 1 (3.4) | 20 (69.0) | 8 (27.6) | |
| IIIa | 29 | 0 (0) | 1 (3.4) | 28 (96.6) | |
| IIIb | 6 | 0 (0) | 0 (0) | 6 (100) | |
| IV | 3 | 0 (0) | 0 (0) | 3 (100) | |
| Lymph node metastasis | | | | | <0.0001 |
| (+) | 36 | 0 (0) | 0 (0) | 36 (100) | |
| (-) | 46 | 6 (13.0) | 29 (63.0) | 11 (24.0) | |
| Liver metastasis | | | | | 0.058 |
| (+) | 7 | 0 (0) | 0 (0) | 7 (100) | |
| (-) | 75 | 6 (8.0) | 29 (38.7) | 40 (53.3) | |

membrane and/or cytoplasm might participate in the metastasis of tumor.

Expression of KL-6 mucin in metastatic lesions

We next examined the expression of KL-6 mucin in lymph node and liver metastatic lesions (Figure 3). For all the 36 cases with lymph node metastasis and all seven cases with liver metastasis, positive staining was observed in metastatic lesions as well as in the primary colorectal carcinoma tissues.

DISCUSSION

MUC1 is abundantly expressed at the surface of epithelial cells in many tissues^[3-6]. MUC1 expression is also observed in carcinomas that arise in several parts of the body, such as the colon, breast, lung, pancreas, papillary thyroid, and gallbladder^[9-13]. The deduced amino acid sequence of MUC1 mucin reveals four distinct domains: the

NH₂-terminal domain consisting of a hydrophobic signal sequence; a highly *O*-glycosylated tandem-repeat domain; a transmembrane domain; and a cytoplasmic domain^[1,2,6]. Previous studies on MUC1 expression in colorectal carcinoma have primarily focused on the tandem-repeat domain, and suggested that tumor cells expressing high levels of MUC1 may have increased invasive and metastatic potential^[10]. However, little is known about the detailed clinicopathological relationship among expression profile of MUC1, metastatic potentiality, and the prognosis for colorectal adenocarcinoma. On the other hand, although the processing of the full length MUC1 core proteins is similar in both normal and tumor cells, they have a remarkable diversity in oligosaccharide moieties^[21,22]. Therefore, we targeted KL-6 mucin, a type of MUC1 bearing sialylated oligosaccharide recognized by KL-6 antibody^[18]. Since sialylation of tumor cell glycoconjugates is thought to contribute to tumor progression and metastasis^[23-26], targeting KL-6 mucin bearing sialylated oligosaccharide seems to be a reasonable strategy.

In our preliminary study, KL-6 mucin was observed in carcinoma cells but not in the surrounding normal cells. However, classification of KL-6 staining evaluated by overall expression level did not show significant relationships between the expression level of KL-6 mucin, metastasis, and patient's prognosis (data not shown). Recently, some reports on breast carcinoma have suggested a significant relationship between metastasis and subcellular location of MUC1 rather than its overall expression level, which led us to focus on the subcellular location of KL-6 mucin in colorectal carcinoma.

In the present study, the circumferential and/or cytoplasmic expression of KL-6 mucin was significantly correlated with lymph node metastasis in colorectal adenocarcinomas (Table 2). In addition, this aberrant localization of KL-6 mucin was likely to participate also in liver metastasis, since all cases having liver metastasis (*n* = 7) showed positive staining in the circumferential membrane and/or cytoplasm (Table 2). It is known that normal epithelial cells release a tailless, soluble form of MUC1 which targets exclusively the apical membrane in tissues^[21]. However, in carcinoma cells with aberrant overexpression of MUC1, this apical polarization is lost, resulting in aberrant localization of MUC1 over the entire cell membrane and in the cytoplasm^[15,16]. It has been proposed that MUC1 mediates anti-adhesion activity by interfering with cell-to-cell and/or cell-to-extracellular matrix interactions, thereby facilitating detachment of tumor cells from the primary growth^[6, 27-29]. This is likely true of KL-6 mucin in colorectal adenocarcinoma, since a high frequency of metastasis was observed in cases showing any aberrant localization of KL-6 mucin (Table 2). This aberrant subcellular expression of KL-6 mucin might facilitate detachment of tumor cells from the primary growth, resulting in an increased ability of the tumor cells to metastasize.

It is notable that all the cases tested showed positive staining in metastatic lesions as well as the primary colorectal carcinoma tissues. Interestingly, in some cases presenting lymph node or liver metastasis, aberrant subcellular localization of KL-6 was observed in only

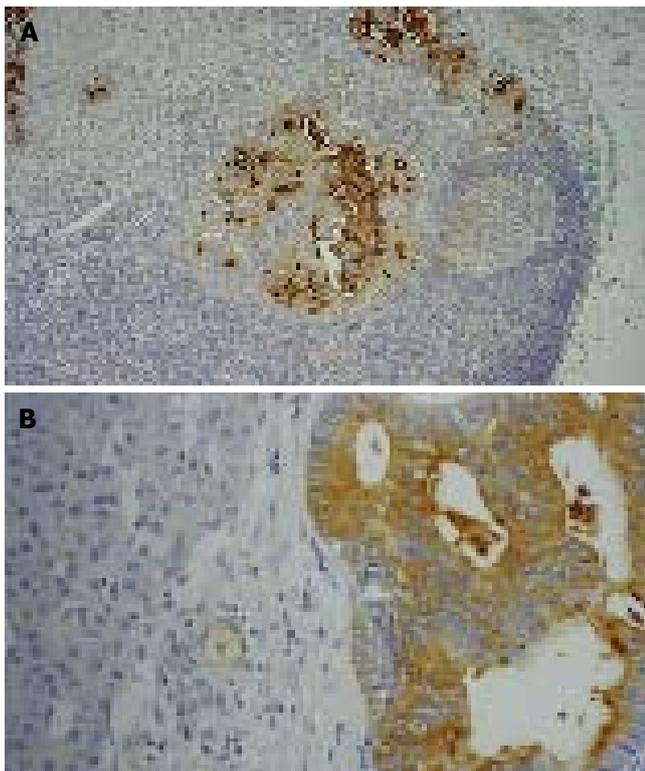


Figure 3 Immunohistochemical staining of metastatic lymph node and liver tissues using KL-6 antibody (x200). **A:** Positive KL-6 expression in a metastatic lymph node; **B:** positive KL-6 expression in metastatic liver tissues.

a few tumor cells (less than 5%) in the primary lesions (data not shown). This suggests that the significant factor is the subcellular location rather than the level of KL-6 expression, and is coincident with a report that breast carcinomas with the cytoplasmic staining pattern, even in a minor focus, are associated with a higher incidence of lymph node metastasis^[17]. However, a considerable number of cases showing positive membranous and/or cytoplasmic staining did not present with metastases. Further investigation is needed to understand the role of KL-6 expression in metastatic events of colorectal adenocarcinoma.

Some studies have reported that, in breast carcinoma, patients expressing MUC1 in the non-apical membranes show worse prognosis than those expressing MUC1 in the apical membrane^[17,30]. Our observation also showed that there was a significant relationship between subcellular location of KL-6 and prognosis in colorectal adenocarcinoma (Figure 2; $P = 0.029$). The five-year survival rates for the cases with apical membrane staining and those with circumferential and/or cytoplasmic staining of KL-6 mucin were 85.7% and 63.0%, respectively (Figure 2). Moreover, our data also showed that cases without KL-6 staining had a better prognosis than those with the apical staining of KL-6 mucin. These data suggest that the expression characteristics of KL-6 mucin would be useful for the prediction of a patient's prognosis in colorectal adenocarcinoma.

In conclusion, subcellular localization of KL-6 mucin plays a crucial role in determining disease outcome and expression of KL-6 mucin in the circumferential

membrane and/or cytoplasm is an important indicator for lymph node and liver metastases as well as the prognosis of patients with colorectal adenocarcinoma.

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COLORECTAL CANCER

Antithrombin reduces reperfusion-induced hepatic metastasis of colon cancer cells

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induced expression of E-selectin through an increase in the endothelial production of PGI₂. These findings also raise the possibility that AT might prevent hepatic metastasis of tumor cells if administered during the resection of liver tumors.

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Key words: Antithrombin; E-selectin; Hepatic ischemia/reperfusion; Metastasis; Prostacyclin; Tumor necrosis factor- α

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Abstract

AIM: To examine whether antithrombin (AT) could prevent hepatic ischemia/reperfusion (I/R)-induced hepatic metastasis by inhibiting tumor necrosis factor (TNF)- α -induced expression of E-selectin in rats.

METHODS: Hepatic I/R was induced in rats and mice by clamping the left branches of the portal vein and the hepatic artery. Cancer cells were injected intrasplenically. The number of metastatic nodules was counted on day 7 after I/R. TNF- α and E-selectin mRNA in hepatic tissue, serum fibrinogen degradation products and hepatic tissue levels of 6-keto-PGF_{1 α} , a stable metabolite of PGI₂, were measured.

RESULTS: AT inhibited increases in hepatic metastasis of tumor cells and hepatic tissue mRNA levels of TNF- α and E-selectin in animals subjected to hepatic I/R. Argatroban, a thrombin inhibitor, did not suppress any of these changes. Both AT and argatroban inhibited I/R-induced coagulation abnormalities. I/R-induced increases of hepatic tissue levels of 6-keto-PGF_{1 α} were significantly enhanced by AT. Pretreatment with indomethacin completely reversed the effects of AT. Administration of OP-2507, a stable PGI₂ analog, showed effects similar to those of AT in this model. Hepatic metastasis in congenital AT-deficient mice subjected to hepatic I/R was significantly increased compared to that observed in wild-type mice. Administration of AT significantly reduced the number of hepatic metastases in congenital AT-deficient mice.

CONCLUSION: AT might reduce I/R-induced hepatic metastasis of colon cancer cells by inhibiting TNF- α -

INTRODUCTION

Hepatic ischemia/reperfusion (I/R) is sometimes induced by temporary clamping of the portal triad during resection of a liver tumor and a portal vein for advanced pancreatic head cancer^[1, 2]. Hepatic I/R may promote hematogenous liver metastases of cancer cells that detach during the surgical procedure^[3]. Endothelial leukocyte adhesion molecules such as E-selectin and intercellular adhesion molecule-1 have been demonstrated to be critically involved in the adhesion of tumor cells to endothelial cells, thereby promoting the metastasis of tumor cells^[4-8]. Expression of these adhesion molecules in endothelial cells is markedly enhanced by pro-inflammatory cytokines such as tumor necrosis factor (TNF)- α , which plays a critical role in the development of I/R-induced liver injury^[9, 10].

Antithrombin (AT), a physiological serine protease inhibitor, plays a critical role in the regulation of the coagulation cascade^[11]. In addition to such regulatory role in the coagulation system, AT exerts an anti-inflammatory activity by increasing the endothelial production of prostacyclin (PGI₂), which is capable of inhibiting TNF- α production by monocytes^[12-14]. According to this anti-inflammatory property, AT reduces I/R-induced liver injury by inhibiting TNF- α production in rats^[15, 16]. These observations suggest that AT might reduce I/R-induced hepatic metastasis of cancer cells

by decreasing the expression of leukocyte adhesion molecules such as E-selectin through an increase in the endothelial production of PGI₂. In the present study, we have examined whether AT could reduce the metastasis of cancer cells injected intrasplenically in rats and congenital AT-deficient mice subjected to hepatic I/R.

MATERIALS AND METHODS

Reagents

AT and argatroban ((2R,4R)4-methyl- $\{N^2-[(3\text{-methyl-1,2,3,4, tetrahydro-8-quinolyl) sulfonyl]-L-arginyl}\}$ -2-piperidinecarboxylic acid monohydrate) were obtained from the Mitsubishi-Welpharma Co., Ltd. (Osaka, Japan). OP-2507 (15 *cis*-(4-*n*-propylcyclohexyl)-16,17,18,19,20-pentanoic-9-deoxy-6,9- α -nitrolo-prostaglandin F₁ methyl ester), a prostacyclin analog, was obtained from the Ono Pharmaceutical Co., Ltd. (Osaka, Japan). Indomethacin (IM), xylazine and RPMI 1640 were purchased from Sigma (St. Louis, MO, USA). Penicillin G, streptomycin, and fetal bovine serum (FBS) were obtained from Invitrogen (Gaithersburg, MD, USA). Ketamine hydrochloride was purchased from Parke-Davis (Morris Plains, NJ, USA). All other reagents were of analytical grade.

Animals

Pathogen-free male Fisher 344 (F344) rats, weighing 180–210 g, and C57BL/6 mice, weighing 18–22 g, were obtained from Clea Japan (Tsukuba, Japan). Congenital AT-deficient mice (AT^{+/-}) were kindly provided by Dr. Kojima, Nagoya University. Plasma levels of AT in AT^{+/-} mice were about 50% of that in wild type^[17]. Care and handling of the animals were in accordance with the National Institute of Health guidelines and the animals were fed standard animal chow and water. All the animal experiments were carried out in a humane manner after receiving permission from the Animal Experiment Committee of the university and in accordance with the Regulation of Animal Experiment and Japanese Governmental Law.

Cell lines

RCN-H4 cells, derived from hepatic metastasis of a F344 rat colon adenocarcinoma cell line, were provided by RIKEN Cell Bank (Tsukuba, Japan)^[18, 19]. RCN-H4 cells were maintained in RPMI 1640 supplemented with 100 mL/L heat inactivated FBS, 100 kU/L penicillin G and 100 mg/L streptomycin in a humidified 950 mL/L air-50 mL/L CO₂ at 37 °C. B16-F10 melanoma cells, which are syngeneic to C57BL/6 mice, were gifted from Cell Resource Center of Tohoku University (Sendai, Japan). B16-F10 cells were maintained in the same medium that was used for RCN-H4 cell culture.

Rat hepatic metastasis model

Under anesthesia with diethyl ether, F344 rats underwent laparotomy. Warm ischemia of the median and left hepatic lobes was induced by clamping the left branches of the portal vein and the hepatic artery. The right lobe was perfused to prevent intestinal congestion. During the period of hepatic ischemia, the rat's abdomen was

covered with a plastic wrap to prevent dehydration. AT (500 U/kg) and OP-2507 (3 µg/kg) were dissolved in double distilled water, and administered intravenously 20 min after ischemia. Argatroban (1 mg/kg) and IM (5 mg/kg) were administered subcutaneously 30 min prior to ischemia. After 30 min of ischemia, the clamp was removed and the right lobe and caudate lobe were resected to prevent shunting to them after reperfusion with 4-0 silk. After resection of the right and caudate lobes, RCN-H4 cells (2×10⁶ cells/rat) were intrasplenically administered using a 30-gauge needle and the wound was closed with 3-0 silk. Sham-operated animals underwent the same operation but without clamping. During the surgical operation, the rat's body temperature was kept between 35.5 °C and 36.5 °C by using a heating pad. The rates of breathing and heart beating were stable during the operation. None of the animals died until the 7th day after the operation.

Mouse hepatic metastasis model

The same surgical procedure as for F344 rats was performed for every mouse except for the anesthesia method (ketamine, 75 mg/kg body weight plus xylazine, 25 mg/kg body weight) and ischemia time (60 min). After resection of the right and the caudate lobes, B16-F10 cells (2×10⁵ cells/mouse) were intrasplenically administered. AT (500 U/kg) was administered 10 min before reperfusion. None of the animals died until the 7th day after the operation.

Calculation of hepatic metastatic nodules

Animals were killed on the 7th day after the operation. The liver was removed, fixed with 10% buffered formalin for a few days, and cut into slices, each about 4 mm thick serially. These slices were stained with hematoxylin-eosin. The number of metastatic nodules was counted under a dissecting microscope (Olympus, Tokyo, Japan) at a magnification of ×100. The liver slice area was calculated with IP Lab software (Solution Systems Co., Ltd.) on a Macintosh computer. The number of metastatic nodules was normalized by the slice area (nodule number/cm²).

Measurement of serum concentration of fibrin and fibrinogen degradation products

Ninety minutes after reperfusion, blood samples were collected in tubes containing 0.1 volume of 38g/L sodium citrate. Blood was centrifuged at 2 000 r/min for 10 min. Serum levels of fibrinogen degradation products (FDP (E)) were measured by a latex agglutination assay, as previously described^[20].

Assay for 6-keto-PGF_{1 α} in liver

Levels of 6-keto-PGF_{1 α} , a stable metabolite of PGI₂, in liver tissue were measured as described previously^[15, 21]. In brief, tissue samples were weighed and homogenized in 0.1 mol/L phosphate buffer (pH 7.4) containing 0.5 g/L sodium azide. The homogenate was centrifuged (2 000 r/min, at 4 °C for 10 min) and the supernatant was stored at -80 °C until measurement. The concentration of 6-keto-PGF_{1 α} was determined with a specific enzyme immunoassay kit (Amersham, Buckinghamshire, UK). The results were expressed as nanograms of 6-keto-PGF_{1 α} per

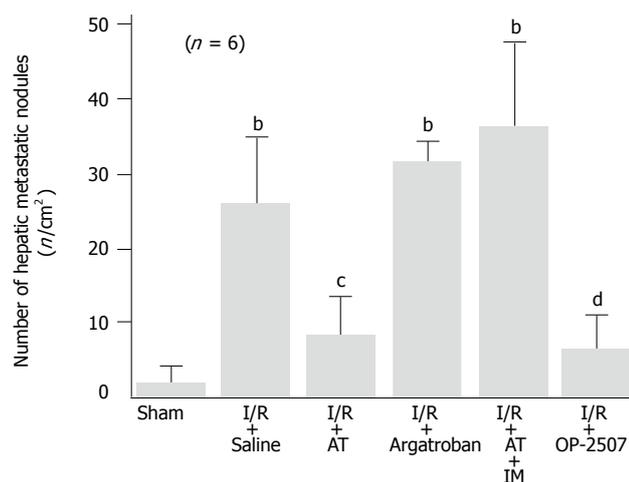


Figure 1 Effects of antithrombin (AT) and various agents on the number of hepatic metastatic nodules in rats after ischemia/reperfusion (I/R). ^b $P < 0.01$ vs sham; ^c $P < 0.05$, ^d $P < 0.01$ vs I/R+saline.

gram tissue.

RNA extraction and cDNA synthesis

Total RNA was isolated after homogenization of the tissue samples in TRIzol reagent according to the manufacturer's instructions. RNA was reverse transcribed into cDNA in a 20- μ L reaction volume containing 2 μ L RNA (1 μ g) in RNase-free water, 2 μ L of 10 \times RT buffer, 4.4 μ L of 25 mmol/L MgCl₂, 4 μ L deoxy NTPs mixture, 1 μ L random hexamers, 0.4 μ L RNase inhibitor, and 0.5 μ L multiscribe reverse transcriptase (50 MU/L). Reverse transcription was performed at 25 °C for 10 min, at 37 °C for 60 min, and at 95 °C for 5 min, followed by quick chilling on ice and storage at 4 °C until subsequent amplification.

Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Real-time quantitative RT-PCR analysis was performed in triplicate using the ABI PRISM 7700 Sequence Detection System instrument and software (Applied Biosystems, Foster City, CA, USA). The primers and probes for the TaqMan system were designed using Primer Express software (Applied Biosystems) and synthesized using PE ABI (Weiterstadt, Germany). The 5'-end nucleotide of the probe was labeled with a reporter dye (FAM). The sequences of the PCR primer sets and probes used for each gene were purchased from TaqMan Gene Expression Assays (Applied Biosystems). The reaction conditions and PCR cycles were set according to the manufacturer's directions.

Statistical analysis

All data were expressed as mean \pm SD. The results were compared using an analysis of variance (ANOVA) followed by Scheffe's test. An associated probability of $P < 0.05$ was considered to be statistically significant.

RESULTS

Effects of AT and argatroban on I/R-induced increase of hepatic metastasis in rats

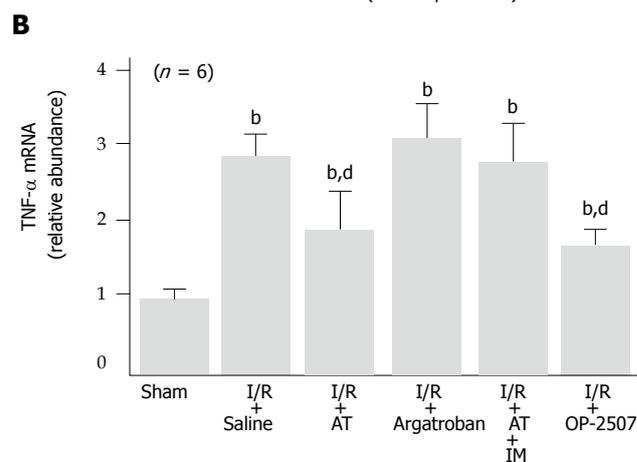
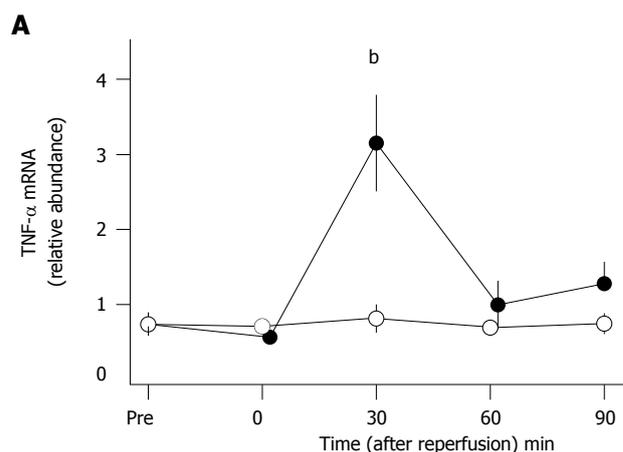


Figure 2 Changes in hepatic tumor necrosis factor (TNF)- α mRNA levels (A) and effects of antithrombin (AT) and various agents on TNF- α mRNA levels (B) in rats subjected to hepatic ischemia/reperfusion (I/R). ^b $P < 0.01$ vs pre-ischemia. ^d $P < 0.01$ vs sham and I/R+saline.

When intrasplenically injected, colon cancer cells formed a significant number of metastatic nodules in the liver of rats subjected to hepatic I/R on d 7 after the injection of cancer cells. The number of metastatic nodules in animals subjected to hepatic I/R was 13.6-fold than that observed in sham-operated animals (Figure 1). AT significantly inhibited the increase in the number of metastatic nodules in animals subjected to hepatic I/R (Figure 1). In contrast, argatroban, a synthetic selective inhibitor for thrombin, had no effect on this increase (Figure 1).

Effects of AT and argatroban on hepatic I/R-induced increase of TNF- α mRNA in hepatic tissue

Hepatic tissue levels of TNF- α mRNA did not significantly increase in sham-operated animals, while these significantly increased 30 min after hepatic I/R and decreased thereafter to pre-ischemia levels (Figure 2A). AT significantly inhibited the I/R-induced increases in the hepatic tissue TNF- α mRNA levels, whereas argatroban did not inhibit these increases (Figure 2B).

Effects of AT and argatroban on hepatic I/R-induced increase of E-selectin mRNA in hepatic tissue

Although hepatic tissue levels of E-selectin mRNA did not increase in sham-operated animals, these levels began

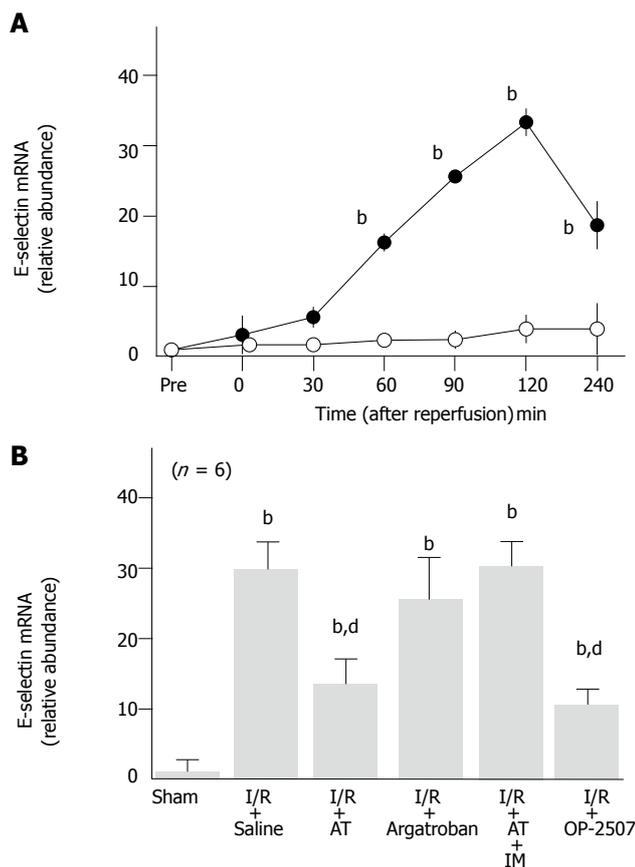


Figure 3 Changes in hepatic E-selectin mRNA levels (A) and effects of antithrombin (AT) and various agents on E-selectin mRNA levels (B) in rats subjected to hepatic ischemia/reperfusion (I/R). ^b $P < 0.01$ vs pre-ischemia. ^d $P < 0.01$ vs sham and I/R+saline.

to increase 60 min after reperfusion, peaking at 120 min after reperfusion in animals subjected to hepatic I/R (Figure 3A). AT significantly inhibited the I/R-induced increases of E-selectin mRNA levels observed in hepatic tissue 120 min after reperfusion, whereas argatroban did not inhibit these increases (Figure 3B).

Effects of AT and argatroban on I/R-induced increase of serum FDP(E)

Serum levels of FDP(E) significantly increased after reperfusion, peaking 90 min after reperfusion in animals subjected to hepatic I/R, while these levels did not increase in sham-operated animals (Figure 4A). Both AT and argatroban inhibited the hepatic I/R-induced increases of serum FDP(E) observed 90 min after reperfusion (Figure 4B).

Effect of AT on hepatic tissue levels of 6-keto-PGF_{1α}

To examine whether AT reduced the hepatic I/R-induced increase in the number of metastatic nodules by promoting the endothelial release of PGI₂, we have analyzed the effect of AT on the hepatic tissue levels of 6-keto-PGF_{1α} after reperfusion. Hepatic tissue levels of 6-keto-PGF_{1α} increased after reperfusion, peaking 90 min after reperfusion (Figure 5A). These levels were significantly higher in the I/R group than in sham-operated animals (Figure 5B). Administration of AT significantly enhanced

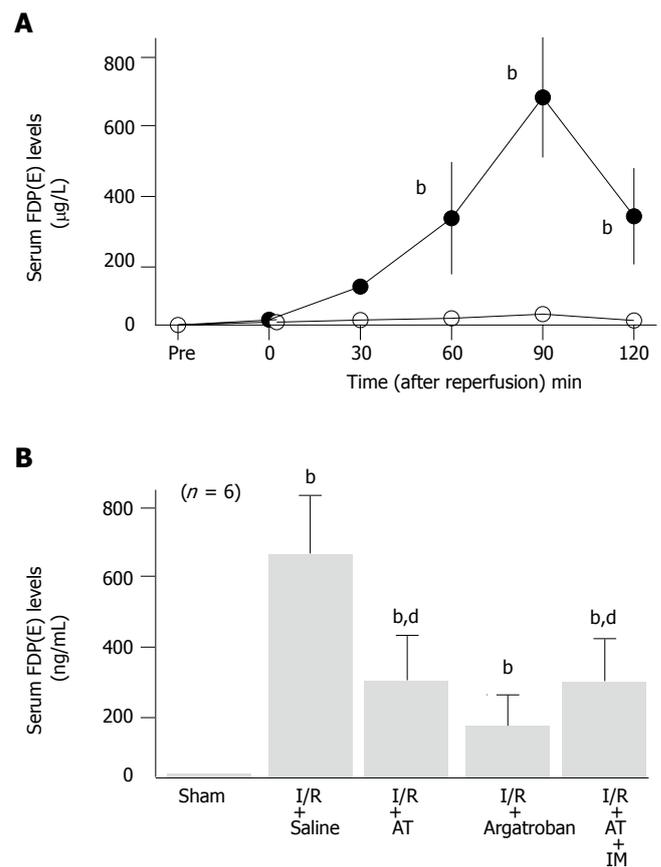


Figure 4 Changes in serum concentrations of fragment E of fibrin and fibrinogen degradation products (FDP(E)) (A) and effects of antithrombin (AT), argatroban and AT+indomethacin (IM) on serum concentrations of FDP(E) (B) in rats after hepatic ischemia/reperfusion (I/R). ^b $P < 0.01$ vs pre-ischemia. ^d $P < 0.01$ vs sham and I/R+saline.

the I/R-induced increases in hepatic levels of 6-keto-PGF_{1α} 90 min after reperfusion, whereas argatroban did not (Figure 5B).

Effect of IM on anti-metastatic effect of AT observed in our experimental model

The anti-metastatic effect of AT was not observed in animals pretreated with IM which is known to inhibit cyclooxygenases (Figure 1). AT did not inhibit hepatic I/R-induced increases of TNF- α and E-selectin mRNA in hepatic tissue from animals pretreated with IM (Figures 2B and 3B). Hepatic I/R-induced increases in serum levels of FDP(E) were inhibited by AT in animals pretreated with IM (Figure 4B). Pretreatment of animals with IM significantly inhibited increases in hepatic tissue levels of 6-keto-PGF_{1α} in animals given AT 90 min after reperfusion (Figure 5B).

Effect of OP-2507 on hepatic I/R-induced increase in the number of metastatic nodules and hepatic tissue levels of TNF- α and E-selectin mRNA

To determine whether PGI₂ inhibited I/R-induced increase in the number of hepatic metastases, we have examined the effect of OP-2507 on the I/R-induced increase in the number of hepatic metastatic nodules. As shown in Figure 1, the increase in the number of metastatic nodules in

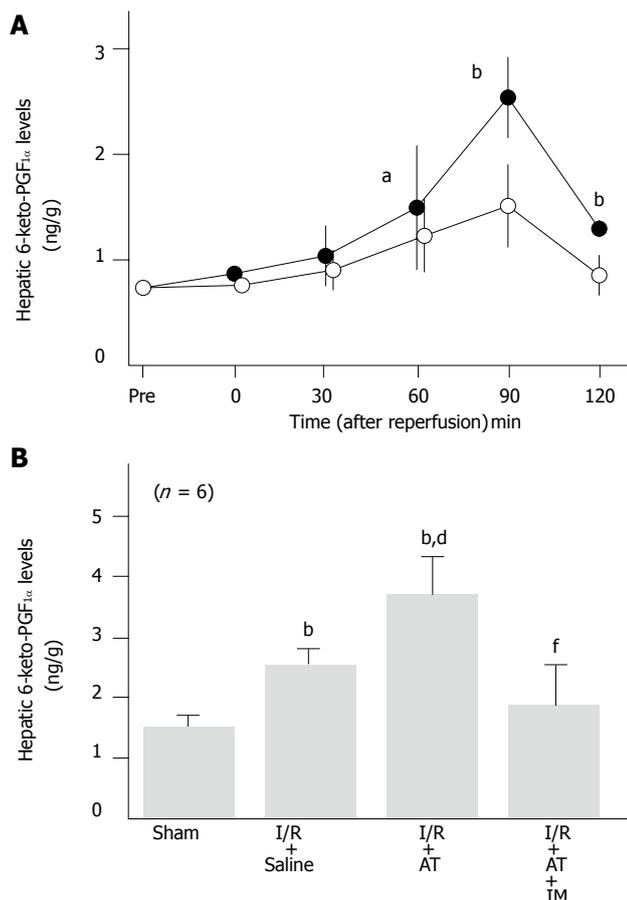


Figure 5 Changes in hepatic 6-keto-PGF_{1α} levels (A) and effects of antithrombin (AT) and AT plus indomethacin (IM) on hepatic 6-keto-PGF_{1α} levels (B) in rats subjected to hepatic ischemia/reperfusion (I/R). ^a*P*<0.05, ^b*P*<0.01 vs pre-ischemia. ^c*P*<0.01 vs sham. ^d*P*<0.01 vs I/R+saline and I/R+AT.

animals subjected to hepatic I/R was significantly inhibited by OP-2507. OP-2507 inhibited the I/R-induced increases in hepatic tissue levels of TNF- α and E-selectin mRNA observed 30 and 120 min after reperfusion, respectively (Figure 2B and 3B).

Hepatic I/R-induced metastasis of B16-F10 cells in congenital AT-deficient mice

To know whether endogenous AT also inhibited hepatic metastasis of B16-F10 cells injected intrasplenically, we have compared the number of hepatic metastatic nodules observed after I/R in congenital AT-deficient mice (AT^{+/-}) with that observed in wild-type C57BL/6J mice. The hepatic I/R-induced increase in the number of hepatic metastatic nodules observed 7 d after reperfusion was significantly higher in congenital AT^{+/-} mice than in wild type mice (Figure 6). AT decreased the number of hepatic metastatic nodules in AT^{+/-} mice subjected to hepatic I/R (Figure 6).

DISCUSSION

In the present study, AT reduced the hepatic I/R-induced increase of hepatic metastasis in rats to which colon cancer cells were intrasplenically injected. TNF- α is a pro-inflammatory cytokine that increases the expression

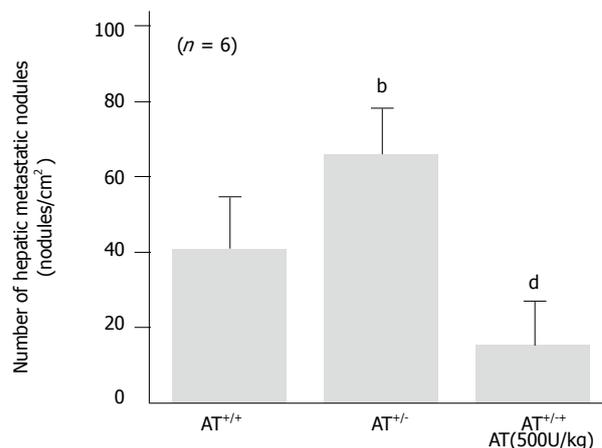


Figure 6 The number of hepatic metastatic nodules in congenital antithrombin (AT)-deficient mice. ^b*P*<0.01 vs AT^{+/+}. ^d*P*<0.01 vs AT^{+/-}.

of leukocyte adhesion molecules such as E-selectin, thereby contributing to the lodgment of cancer cells on the sinusoidal surface, leading to metastatic nodule formation^[22]. AT inhibited hepatic I/R-induced increases in hepatic tissue levels of TNF- α and E-selectin mRNA, suggesting that AT might inhibit the hepatic I/R-induced increase of colon cancer cells metastases in our rat model by inhibiting TNF- α production. TNF- α plays a critical role in microthrombus formation in hepatic I/R^[23]. Since thrombin increases the expression of E-selectin by activating protease-activated receptor-1 on endothelial cells^[24], AT might inhibit the hepatic metastasis of colon cancer cells by inhibiting thrombin activity. However, this possibility seemed less likely, since argatroban, a selective inhibitor of thrombin that inhibited hepatic I/R-induced increases of serum FDP (E) to the same extent as AT, did not inhibit the development of hepatic metastases in the present study. We have previously reported that AT inhibits hepatic I/R-induced increases in hepatic tissue levels of TNF- α by promoting the I/R-induced increase in endothelial production of PGI₂^[16]. Consistent with this report is the present observation demonstrating that AT enhanced the hepatic I/R-induced increases in hepatic tissue levels of 6-keto-PGF_{1α}. Pretreatment with IM reversed the effects of AT, including the inhibition of metastasis and increases in hepatic tissue levels of both TNF- α and E-selectin mRNA, suggesting that AT might inhibit hepatic I/R-induced increase in the metastasis of colon cancer cells by increasing endothelial production of PGI₂. Furthermore, OP-2507, a stable derivative of PGI₂, showed effects similar to those of AT, supporting the hypothesis described above.

Hepatic metastasis of intrasplenically injected cancer cells was significantly increased in heterozygous congenital AT-deficient mice whose plasma AT level is about half that of wild type mice^[17] and replacement of AT in congenital AT-deficient mice reversed this increase in hepatic metastasis in the present study. These observations suggest that endogenous AT as well as AT given intravenously might be critically involved in the inhibition of hepatic I/R-induced increase of hepatic metastases in our experimental model.

Although the findings of the present study strongly

suggest that AT might inhibit the metastasis of colon cancer cells via the promotion of endothelial production of PGI₂, it is possible that a direct inhibition of tumor cell growth by AT also contribute to the inhibition of hepatic I/R-induced increase of colon cancer cell metastases. Our preliminary experiments using the monotetrazolium assay demonstrated that AT (5 kU/L) significantly inhibits the growth of RCN-H4 cells after incubation for 48 h^[25]. Therefore, although IM pretreatment abrogated the anti-metastatic effect of AT in the present study, a direct effect of AT on cancer cell growth might contribute, at least in part, to the anti-metastatic effect of AT. This possibility should be further examined in future experiments.

AT concentrates are currently available for the treatment of disseminated intravascular coagulation and thrombosis in the clinical setting^[26-28]. The findings of the present study raise the possibility that AT concentrates can effectively prevent hepatic metastasis of cancer cells during hepatectomy. These possibilities should be further examined in the clinical setting in the near future.

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Helicobacter pylori

Discrepancies between primary physician practice and treatment guidelines for *Helicobacter pylori* infection in Korea

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Abstract

AIM: To evaluate the attitude of primary care physicians in the diagnosis and treatment of *Helicobacter pylori* (*H pylori*) infection.

METHODS: Primary care physicians in the Seoul metropolitan area answered self-administered questionnaire from January to March 2003.

RESULTS: One hundred and eight doctors responded to the questionnaire. The most frequent reasons for testing *H pylori* infection were gastric and duodenal ulcers (93.5% and 88.9%, respectively). For patients with *H pylori* positive dyspepsia, 28.7% of doctors always tried to eradicate the worm and 34.4% treated selectively. A large proportion (28.7%) of primary care physicians treated *H pylori* on a patient's request basis. Only 9.3% of primary care physicians always conducted follow-up testing after treating *H pylori* infection. When *H pylori* was not cleared by the first treatment, 40.7% of doctors reused the same regimen, 16.7% changed to another triple regimen and 25% to a quadruple regimen.

CONCLUSION: It has been well documented that the issuance of guidelines alone has little impact on practice. Communication between primary care physicians and gastroenterologists in the form of continuous medical education is required.

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Key words: *Helicobacter pylori*; Guidelines; Primary care

INTRODUCTION

It is now accepted that infection of the gastric mucosa with *Helicobacter pylori* (*H pylori*) causes chronic gastritis. More than 90% of peptic ulcer patients are infected with *H pylori* and it has been shown that successful treatment prevents ulcer relapse^[1-3]. National and international guidelines have been published on the management of *H pylori*, and it is tacitly assumed that these guidelines are adhered to in clinical practice^[4-7]. But, there seems to be a discrepancy between current testing and treatment guidelines and clinical practice^[8,9]. In particular, the approaches used in primary practice may differ markedly from those used in referral hospitals^[10-13]. Patients commonly visit both primary care physicians and gastroenterologists because of upper gastrointestinal symptoms. Variable *H pylori* detection methods, including serologic assays and the urea breath test, are currently used. Although most physicians do not believe that *H pylori* causes non-ulcer dyspepsia, the majority often prescribe antibiotics for *H pylori* eradication. The extent to which research findings regarding *H pylori* have modified physicians' practice in general has not been well studied. We conducted the present study to determine whether current guidelines regarding *H pylori* infection have influenced diagnostic and therapeutic primary care practice.

MATERIALS AND METHODS

From January to March 2003, we conducted an observational, transverse study by using a self-administered questionnaire. Primary care physicians in the metropolitan area of Seoul were randomly selected from the membership database of the Seoul Medical Association. Questionnaires were distributed by post, and non-respondents were sent reminders and then contacted by telephone. One hundred and thirty-five doctors participated in the study.

Table 1 Korean guidelines for *H pylori* treatment (Korean *H pylori* Study Group, 1998)

| | |
|--|--|
| Indication for <i>H pylori</i> eradication | Peptic ulcer Regardless of the stage of ulcer Low-grade MALT associated lymphoma Stage IE1 After endoscopic mucosal resection (EMR) of early gastric cancer (EGC) |
| Recommended first line therapy | PPI-based triple therapy for 1-2 wk - PPI (omeprazole 20 mg or lansoprazole 30 mg or pantoprazole 40 mg) b.i.d. - Amoxicillin (not ampicillin) 1 000 mg b.i.d. - Clarithromycin (or metronidazole) 500 mg b.i.d. |
| Follow-up after eradication therapy | Urea breath test: test of choice, if available Or both biopsy urease test and histology At least 4 wk after completion of therapy Serology: not useful to confirm the eradication |
| Recommended second-line therapy | Quadruple therapy for 1 wk (PPI+conventional bismuth-based triple therapy) - PPI (omeprazole 20 mg or lansoprazole 30 mg or pantoprazole 40 mg) b.i.d. - Denol 120 mg b.i.d. - Metronidazole 400-500 mg t.i.d. - Tetracycline 500 mg q.i.d. |

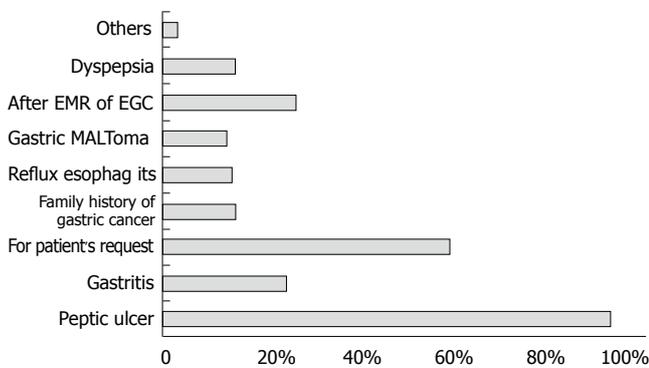


Figure 1 When do you test for *H pylori*?

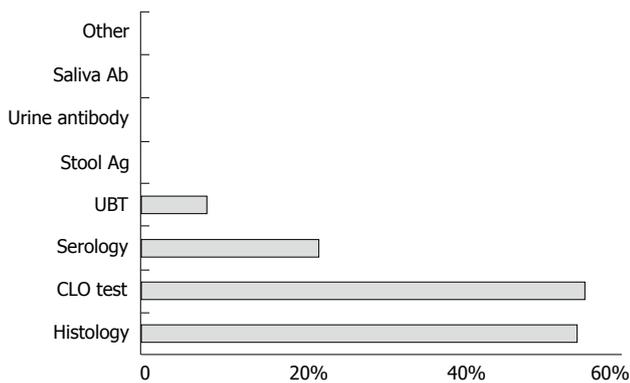


Figure 2 Which test do you prefer?

RESULTS

One hundred and eight doctors (80%) responded to the survey. We itemized below the physicians' responses to the questions given in the questionnaire.

When do you test for *H pylori*?

Primary care physicians widely used the *H pylori* test in cases of gastric ulcer and duodenal ulcer (88.9% and 93.5%, respectively, Figure 1). But they conducted tests for *H pylori* only in 26.9% and 13% of patients, after surgery

for early gastric cancer or Maltoma (Figure 1). Frequently physicians tested for *H pylori* in cases of gastritis and due to a patient's request (25.0% and 58.3%, respectively, Figure 1).

Which test do you prefer?

More than half of the primary care physicians stated that they used the rapid urease test and biopsy, (55.6% and 54.6%, respectively, Figure 2), and a minority used the urea breath test and serology (8.3% and 22.2%, respectively, Figure 2).

How do you eradicate *H pylori* infection?

An 88% of physicians responded that they prescribed a proton pump inhibitor (PPI)-based triple regimen according to Korean guidelines (Table 1, Figure 3), and only small numbers were found to prescribed dual and quadruple regimens (2.8% and 2.8%, respectively, Figure 3).

How long do you treat *H pylori* infection?

The majority of physicians responded that they prescribe medication for 7,14 d (90.7%), according to Korean guidelines (Figure 4). A small number responded that they prescribed for less than 7, 21 or 28 d, accounting for 4.6%, 1.9%, 2.8%, respectively (Figure 4).

Do you perform follow-up testing after treating *H pylori* infection?

Only 9.3% replied that they always conducted follow-up testing after treating *H pylori* infection (Figure 5). The majority of primary care physicians stated that they selectively apply follow-up tests (Figure 5).

What kind of method do you prefer as follow-up test?

The majority percent of physicians stated that they used the rapid urease test or biopsy, accounting for 35.2% and 25.9%, respectively (Figure 6), while a minority favored urea breath testing or serology, accounting for 21.3% and 6.5%, respectively (Figure 6).

Treatment plan after failure to eradicate *H pylori*

Surprisingly, a large proportion (40.7%) of primary care physicians prescribed the original regimen after failure to eradicate *H pylori* (Figure 7). Only 25.0% physicians

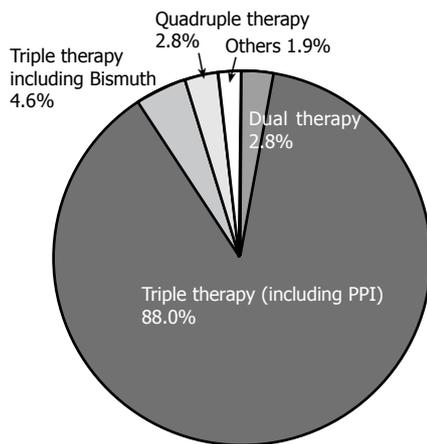


Figure 3 How do you eradicate *H. pylori* infection?

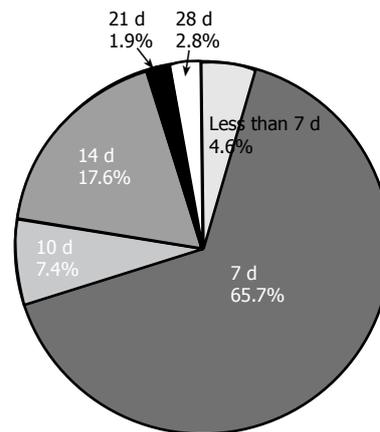


Figure 4 How long do you treat *H. pylori* infection?

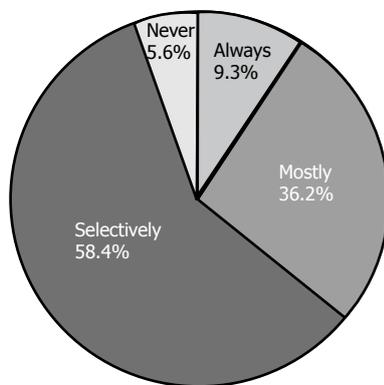


Figure 5 Do you perform follow-up testing after treating *H. pylori* infection?

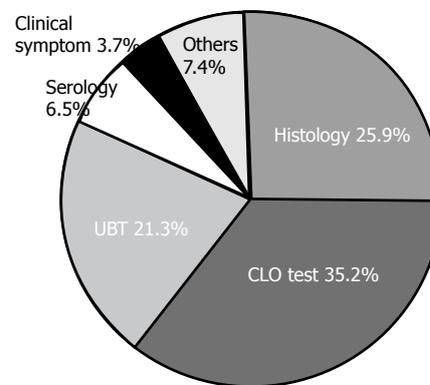


Figure 6 What kind of method do you prefer as follow-up test?

prescribed regimens complying with Korean guidelines (Figure 7).

Do you treat *H. pylori* infection in dyspepsia patients without peptic ulcer?

Physicians (31.5%) responded that they always eradicate *H. pylori* (Figure 8), while 10.2% treated *H. pylori* on patient's request without testing (Figure 8).

Do you medicate for *H. pylori* eradication by patient's request?

A large number (28.7%) of physicians responded positively (Figure 9).

DISCUSSION

The prevalence of *H. pylori* among Korean adults is 60%-80%^[14], and gastric cancer remains the most common malignancy in Korea^[15]. Most Korean primary care physicians are interested in *H. pylori*, and more frequently prescribe medication for the treatment of *H. pylori* than gastroenterologist. The indications for *H. pylori* eradication in Korea (Korean *H. pylori* Study Group, 1998) are peptic ulcer, low-grade MALT-associated lymphoma, and post-endoscopic mucosal resection of early gastric cancer (Table 1). The recommended first-line therapy in Korea is the PPI-based triple therapy for 1 to

2 weeks. PPI would be the omeprazole, lansoprazole, or pantoprazole (Table 1). The treatment of choice in terms of antibiotics is the amoxicillin+clarithromycin (Table 1). The recommended second-line therapy according to the Korean guidelines is quadruple therapy for a week (Table 1), based on PPI, Denol, metronidazole, and tetracycline (Table 1). The issued Korean guidelines are similar to those issued by the Asia Pacific Consensus Conference on the management of *H. pylori* infection^[6]. The survey conducted for the present study involved mailing a questionnaire to primary physicians in Seoul, Korea. The questions primarily addressed physician decisions concerning the evaluation and treatment of *H. pylori* infection in patients with gastroenterologic disease. Alternative treatment regimens were also examined. The survey results indicate that primary care physicians widely adopt *H. pylori* testing in cases of gastric ulcer and duodenal ulcer according to Korean guidelines. However, physicians only conduct *H. pylori* testing in 26.9% and 13.0% of postoperative early gastric cancer and Maltoma patients. In addition, they frequently test for *H. pylori* in cases of gastritis and due to a patient's request, accounting for 25.0% and 58.3%, respectively, but many do not perform follow-up testing after *H. pylori* treatment. Only 9.3% of primary care physicians always conduct follow-up testing after *H. pylori* treatment. The majority of primary care physicians prefer the rapid urease test

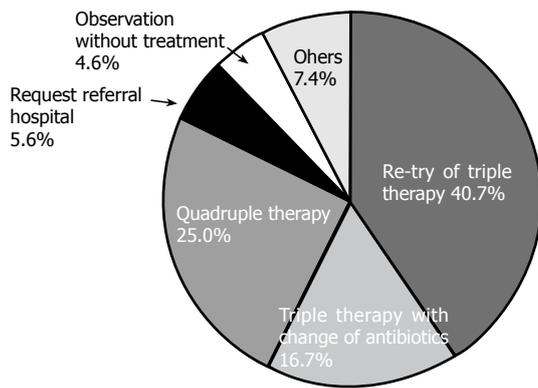


Figure 7 Treatment plan after failure to eradicate *H pylori*.

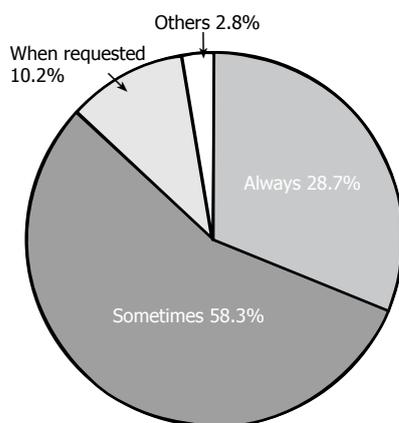


Figure 8 Do you treat *H pylori* infection in dyspepsia patients without peptic ulcer?

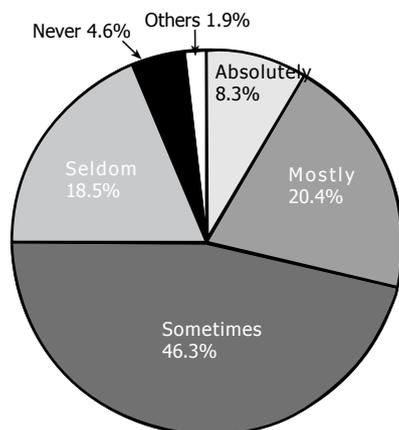


Figure 9 Do you medicate for *H pylori* eradication by patient's request?

or biopsy, accounting for 35.2%, 25.9%, respectively, as follow-up tests, because generally in Korea primary care physicians have an endoscopic unit, but not urea breath test equipment; 6.5% physicians use a serology-based follow-up test. Only 25.0% prescribe a quadruple regimen as second line therapy, contrary to the Korean guidelines and a large number (40.7%) of physicians prescribe the same regimen after failing to eradicate *H pylori*. In addition, they frequently treat *H pylori* in cases of non-ulcer

dyspepsia and patient's request. This finding is at odds with the current guideline and primary care practice for the diagnosis and treatment of *H pylori* infection in Korea. Moreover, the finding of the present study compare well with data published in other countries^[10-13]. Thus, the issuance of guidelines has little impact on practice. Our findings suggest that communication programs, such as continuous medical education, between primary care physicians and gastroenterologists are needed. Moreover, schemes designed to ensure guideline implementation should be preceded by a detailed analysis of likely primary care physician response.

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CLINICAL RESEARCH

Gallbladder bile composition in patients with Crohn's disease

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Abstract

AIM: To further elucidate the pathogenesis and mechanisms of the high risk of gallstone formation in Crohn's disease.

METHODS: Gallbladder bile was obtained from patients with Crohn's disease who were admitted for elective surgery (17 with ileal/ileocolonic disease and 7 with Crohn's colitis). Fourteen gallstone patients served as controls. Duodenal bile was obtained from ten healthy subjects before and after the treatment with ursodeoxycholic acid. Bile was analyzed for biliary lipids, bile acids, bilirubin, crystals, and crystal detection time (CDT). Cholesterol saturation index was calculated.

RESULTS: The biliary concentration of bilirubin was about 50% higher in patients with Crohn's disease than in patients with cholesterol gallstones. Ten of the patients with Crohn's disease involving ileum and three of those with Crohn's colitis had cholesterol saturated bile. Four patients with ileal disease and one of those with colonic disease displayed cholesterol crystals in their bile. About 1/3 of the patients with Crohn's disease had a short CDT. Treatment of healthy subjects with ursodeoxycholic acid did not increase the concentration of bilirubin in duodenal bile. Several patients with Crohn's disease, with or without ileal resection/disease had gallbladder bile supersaturated with cholesterol and short CDT and contained cholesterol crystals. The biliary concentration of bilirubin was also increased in patients with Crohn's colitis probably not due to bile acid malabsorption.

CONCLUSION: Several factors may be of importance for the high risk of developing gallstones of both cholesterol and pigment types in patients with Crohn's disease.

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INTRODUCTION

The prevalence of gallstone disease in patients with Crohn's disease is about two-fold higher than that in general population^[1-8]. This is true not only for patients with ileal disease/resection but also for patients with Crohn's colitis. No large studies of gallstone composition are available in patients with Crohn's disease but in two small series, while both pigment stones and cholesterol-rich stones have been reported^[9,10]. The pathogenesis of gallstones in patients with Crohn's disease still remains to be elucidated.

One hypothesis for the increased prevalence of gallstone disease in patients with Crohn's disease is that the bile acid malabsorption in patients with diseased or resected ileum may lead to cholesterol supersaturated bile. In fact, supersaturated bile has been reported in some^[11-14] but not all studies of patients with ileal disease or resection^[8-10,15-19].

Another hypothesis for gallstone formation in patients with Crohn's disease is that patients with ileal disease or resection develop pigment stones as a consequence of increased spillage of malabsorbed bile acids into the colon where they solubilize unconjugated bilirubin and promote its absorption and thereby increase the rate of bilirubin secretion into the bile. In support of this hypothesis, an increased concentration of bilirubin in gallbladder bile or duodenal bile of patients with chronic ileitis or previous ileectomy has been reported^[10,14,17,18]. Brink *et al.*^[20] demonstrated that bile acid malabsorption after ileectomy of rats induces enterohepatic circulation of bilirubin and doubles the secretion rate of bilirubin into the bile. The same research group has also shown that adding ursodeoxycholic acid to the diet of mice and rats can increase the cecal bile acid levels and bilirubin secretion rates into the bile probably by inducing enterohepatic cycling of bilirubin^[21].

The aims of the present study were to determine the biliary lipid composition, occurrence of cholesterol crystals, crystallization time, and bilirubin concentration in gallbladder bile of patients with ileal Crohn's disease

Table 1 Characteristics of patients with Crohn's disease and controls with gallstone disease

| Patient group | n | Sex (M/F) | Mean age (range) (yr) | Previous surgery | Present surgery | Medical treatment |
|------------------------|----|-----------|-----------------------|---|--|---|
| Crohn's disease | | | | | | |
| Ileitis or ileocolitis | 17 | 8/9 | 38 (23-70) | Ileal or ileocolonic resection (n = 12) | Colonic or ileocolonic resection (n = 15) Cholecystectomy (n = 2) | Steroids (n = 11) Azathioprine (n = 4) Nitromidazole (n = 6) 5-ASA (n = 2) |
| Colitis | 7 | 5/2 | 42 (28-57) | Partial colonic resection (n = 3) | Partial colonic resection or colectomy (n = 7) | Steroids (n = 5) Azathioprine (n = 1) Nitromidazole (n = 3) |
| Gallstone disease | 14 | 1/13 | 47 (27-57) | | Cholecystectomy (n = 14) | |

with or without the involvement of colon and especially patients with Crohn's colitis in comparison to patients with cholesterol gallstone disease undergoing cholecystectomy served as control group and to study the influence of treatment with ursodeoxycholic acid on the duodenal concentration of bilirubin in human subjects.

MATERIALS AND METHODS

Patients and healthy subjects

Twenty-four patients with Crohn's disease admitted for elective surgery were included in the study. Clinical details are given in (Table 1). One patient had slightly elevated serum alkaline phosphatase level and three had slightly elevated transaminase and/or gamma glutamyl transpeptidase (GGT)-level. All other patients had normal laboratory tests of liver function including serum bilirubin. Seventeen patients had ileal or ileocolonic disease and 7 patients had Crohn's disease confined solely to the colon. Two patients with gallstones were cholecystectomized, one of them simultaneously underwent colectomy. All other patients were admitted for ileal, ileocolonic or colonic resection due to the failure of pharmacological treatment.

Fourteen consecutive patients with cholesterol gallstones admitted for cholecystectomy served as controls (Table 1).

In another experiment, 10 healthy subjects (6 men and 4 women, mean age 44 years) were studied before and after the treatment with ursodeoxycholic acid.

Informed consent was obtained from all the participants. The ethical aspect of the study was approved by the Ethical Committee of Karolinska Hospital Huddinge.

Experimental procedures

After the abdomen was opened, bile from the gallbladder was obtained by needle aspiration. The bile was collected in sterile tubes surrounded by foil and sent to the laboratory for analysis.

The healthy subjects were treated with ursodeoxycholic acid (Ursofalk® in 250 mg capsules, obtained from Dr Falk Pharma, Freiburg, Germany) at a daily dose of 15 mg/kg for 3 wk. Before and after the treatment, the bile was collected with an oroduodenal tube in the morning after an overnight fast. Gallbladder contraction was stimulated by an intravenous injection of cholecystokinin and 5-10 mL of the concentrated bile was obtained through the tube. The bile was collected in a test tube surrounded by a foil and was sent to the laboratory for analysis. Serum samples

were also collected for analysis of bilirubin.

Biliary bilirubin concentration

An aliquot of the bile was immediately diluted with saline and the bilirubin concentration was determined by a similar procedure as for serum bilirubin as described previously^[18]. The biliary concentration was expressed as mmol/L in gallbladder bile and as micromoles of bilirubin per millimole bile acid in duodenal bile.

Biliary lipids and bile acid composition

A portion of the gallbladder bile was immediately extracted with 20 volumes of chloroform-methanol 2:1 (vol/vol) and analyzed for cholesterol and phospholipids. Cholesterol was determined by an enzymatic method^[22] and phospholipids by the method of Rouser *et al.*^[23] The total bile acid concentration in one aliquot of the bile sample was determined using 3-alpha-hydroxy steroid dehydrogenase assay^[24]. The relative concentration of cholesterol bile acids and phospholipids was expressed as molar percentage of the total biliary lipids. The cholesterol saturation was calculated according to Carey^[25]. Bile acid composition was determined using gas-liquid chromatography^[26].

Analysis of cholesterol crystals and crystallization time (CDT)

Gallbladder bile samples were examined for typical rhomboid monohydrate cholesterol crystals by polarizing light microscopy on pre-heated slides. CDT was determined by the method of Holan *et al.*^[27] with minor modifications^[28]. After centrifugation of about 6 mL bile at 100 000 g for 2 h, 3 mL from the middle phase was transferred into a sterile glass vial and sealed with a cap equipped with permeable silicon membrane. The vial was stored in darkness in an incubator at 37 °C. About 3 µL from the top, middle and bottom portions was aspirated each day, mixed and placed on a pre-heated slide and viewed thoroughly by polarizing light microscopy. CDT was defined as the number of days until the appearance of typical rhomboid monohydrate cholesterol crystals.

Statistical analysis

Data were given as mean ± SE. Comparisons of the data between patients and healthy subjects were calculated using Mann-Whitney's rank sum test and Wilcoxon's sum of rank test. $P < 0.05$ was considered statistically significant.

Table 2 Biliary lipid composition and cholesterol saturation (mean \pm SE)

| | Crohn's disease ileitis or ileocolitis (n = 17) | Crohn's disease colitis (n = 7) | Crohn's disease all patients (n = 24) | Gallstone disease (n = 14) |
|----------------------------|---|---------------------------------|---------------------------------------|----------------------------|
| Cholesterol (molar%) | 7.4 \pm 0.7 | 6.4 \pm 0.9 | 7.1 \pm 0.6 | 9.8 \pm 1.2 |
| Phospholipids (molar%) | 22.3 \pm 1.2 | 25.0 \pm 1.8 | 23.1 \pm 1.0 | 25.1 \pm 1.8 |
| Bile acids (molar%) | 70.3 \pm 1.6 | 68.6 \pm 2.4 | 69.8 \pm 1.3 | 65.1 \pm 3.0 |
| Cholesterol saturation (%) | 103 \pm 9 | 83 \pm 9 ^a | 97 \pm 7 | 138 \pm 18 |

^aP < 0.05 vs patients with gallstone disease.

Table 3 Biliary bile acid composition (mean \pm SE)

| | Crohn's disease ileitis or ileocolitis (n = 17) | Crohn's disease colitis (n = 7) | Crohn's disease all patients (n = 24) | Gallstone disease (n = 14) |
|---------------------------|---|---------------------------------|---------------------------------------|----------------------------|
| Cholic acid (%) | 44.5 \pm 3.3 | 43.2 \pm 2.2 | 44.1 \pm 2.4 ^a | 35.4 \pm 2.7 |
| Chenodeoxycholic acid (%) | 43.0 \pm 2.9 ^b | 45.3 \pm 3.8 ^b | 43.7 \pm 2.3 ^b | 31.2 \pm 2.4 |
| Deoxycholic acid (%) | 8.4 \pm 2.3 ^c | 10.7 \pm 3.2 ^c | 9.1 \pm 1.8 ^c | 30.7 \pm 4.3 |
| Lithocholic acid (%) | 0.10 \pm 0.09 ^f | 0.01 \pm 0.01 ^c | 0.07 \pm 0.07 ^e | 1.5 \pm 0.2 |
| Ursodeoxycholic acid (%) | 3.6 \pm 1.8 | 0.7 \pm 0.5 | 2.7 \pm 1.3 | 1.2 \pm 0.3 |

P < 0.05, ²P < 0.01, ³P < 0.001 vs patients with gallstone disease.

Table 4 Bilirubin concentrations in bile (mean \pm SE)

| | Crohn's disease ileitis or ileocolitis (n = 15) | Crohn's disease colitis (n = 6) | Crohn's disease all patients (n = 21) | Gallstone disease (n = 14) |
|--------------------|---|---------------------------------|---------------------------------------|----------------------------|
| Bilirubin (mmol/L) | 4.6 \pm 0.7 ^a | 5.9 \pm 1.6 | 5.0 \pm 0.7 | 2.6 \pm 0.2 |

* Significantly different from corresponding value of patients with gallstone disease. ^aP < 0.05 vs patients with gallstone disease.

RESULTS

Gall bladder bile composition

Data on biliary lipid composition are given in (Table 2). The cholesterol saturation of bile was significantly lower in patients with Crohn's disease confined to the colon than in patients with the gallstone. In contrast, 10 out of 17 patients with Crohn's disease involving the ileum had cholesterol-saturated bile. Nevertheless, all patients with CD as a group tended to have lower cholesterol saturation compared to patients with gallstone ($P = 0.055$).

Bile acid composition is shown in (Table 3). Cholic acid, chenodeoxycholic acid and deoxycholic acid were the dominant bile acids both in patients with Crohn's disease and in patients with gallstone. The patients with Crohn's disease had significantly lower proportions of deoxycholic acid and lithocholic acid than the patients with gallstone. The proportions of cholic acid and chenodeoxycholic acid were concomitantly increased.

The biliary bilirubin concentration was about 50% higher in patients with Crohn's disease than in patients with gallstone (Table 4). No difference was obtained between patients with Crohn's disease confined to the colon and those with ileal involvement. The bilirubin concentration tended to be higher in patients with Crohn's disease

confined to the colon in patients with ileal involvement but the difference did not reach statistical significance.

Cholesterol crystals and CDT

Cholesterol crystals were present in four gallbladder samples of the 17 patients with Crohn's disease involving the ileum. One of the patients with cholesterol crystals also had gallstones and was cholecystectomized. The gallbladder bile was saturated with cholesterol. Only one of the patients with Crohn's colitis displayed cholesterol crystals. Also this patient had gallstones in saturated bile and was cholecystectomized. Most of the patients (9 out of 14) with cholesterol gallstones displayed cholesterol crystals.

CDT was measured only in patients with Crohn's disease. Six out of sixteen patients with ileal involvement had a short CDT (mean 4 d, range 1-7 d). Two of six patients with Crohn's colitis also had a short CDT (4 and 5 d, respectively).

Treatment with ursodeoxycholic acid

The results are summarized in (Table 5). Ursodeoxycholic acid accounted for (0.5 \pm 0.5)% of the total biliary bile acids before the treatment. Treatment with ursodeoxycholic acid increased the bile acid to (54.8 \pm 3.8)%. Cholic acid, chenodeoxycholic acid, and deoxycholic acid were concomitantly decreased. Treatment with ursodeoxycholic acid decreased the cholesterol saturation from (101 \pm 10)% to (52 \pm 5)%. The biliary bilirubin concentration expressed as mmol/mol bile acid did not change after the treatment with ursodeoxycholic acid. The serum concentration of bilirubin also did not change after the treatment with ursodeoxycholic acid.

DISCUSSION

Several studies have shown that patients with ileal Crohn's

Table 5 Data on healthy subjects treated with ursodeoxycholic acid (UDCA)

| Patient s | Sex | Age (yr) | BMI | Cholesterol saturation (%) | | Cholesterol (molar %) | | Bile acids (molar %) | | UDCA i (%) | | Bile acids (mmol/mL) | | Bilirubin (mmol/L) | | Bilirubin/bile acids (mmol/mol) | |
|--------------|-----|-------------|------|-------------------------------|-----------------|--------------------------|------------------|-------------------------|-------------------|---------------|-------------------|-------------------------|-------|-----------------------|-------|------------------------------------|------|
| | | | | A | B | A | B | A | B | A | B | A | B | A | B | A | B |
| 1 | F | 39 | 22.2 | 103 | 31 | 6.6 | 1.7 | 74.5 | 82.8 | 0.0 | 49.9 | 57.1 | 100.9 | 490 | 1 058 | 8.6 | 10.5 |
| 2 | F | 40 | 24.4 | 74 | 38 | 4.8 | 2.4 | 75.8 | 79.2 | 0.0 | 65.3 | 92.8 | 95.6 | 1 466 | 571 | 15.8 | 6.0 |
| 3 | F | 30 | 26.8 | 116 | 46 | 9.1 | 3.4 | 65.8 | 72.7 | 0.0 | - | 75.4 | 65.8 | 1 031 | 798 | 13.7 | 12.1 |
| 4 | F | 58 | 26.3 | 32 | 42 | 2.4 | 2.8 | 71.7 | 76.1 | 0.0 | 55.8 | 117.8 | 73.6 | 1 265 | 667 | 10.7 | 9.1 |
| 5 | M | 33 | 29.4 | 125 | 51 | 7.4 | 3.8 | 76.2 | 71.4 | 5.2 | 55.1 | 52.1 | 117.4 | 1 045 | 1 111 | 20.0 | 9.5 |
| 6 | M | 30 | 24.0 | 104 | 60 | 6.1 | 3.7 | 77.6 | 78.2 | 0.0 | 66.5 | 97.9 | 32.1 | 1 301 | 418 | 13.3 | 13.0 |
| 7 | M | 34 | 24.9 | 128 | 38 | 8.9 | 2.5 | 70.5 | 76.7 | 0.0 | 54.3 | 52.3 | 26.3 | 993 | 375 | 19.0 | 14.3 |
| 8 | M | 73 | 26.2 | 118 | 55 | 8.9 | 4.3 | 67.5 | 68.6 | 0.0 | 35.7 | 31.0 | 61.9 | 1 168 | 1 497 | 37.7 | 24.2 |
| 9 | M | 49 | 23.2 | 77 | 79 | 5.6 | 4.9 | 71.3 | 77.7 | 0.0 | 41.3 | 33.5 | 12.8 | 662 | 365 | 19.8 | 28.5 |
| 10 | M | 55 | 24.3 | 135 | 77 | 9.8 | 4.9 | 68.6 | 76.7 | 0.0 | 69.7 | 16.4 | 8.5 | 568 | 197 | 34.5 | 23.3 |
| Mean | | 44 | 25.2 | 101 | 52 ^b | 7.0 | 3.4 ^b | 72.0 | 76.0 ^a | 0.5 | 54.8 ^b | 62.6 | 59.5 | 999 | 706 | 19.3 | 15.1 |
| SE | | 4.5 | 0.7 | 10 | 5 | 0.7 | 0.3 | 1.26 | 1.3 | 0.5 | 3.8 | 10.3 | 12.1 | 104 | 130 | 3.1 | 2.4 |

A = before UDCA feeding; B = after UDCA feeding; ^aP < 0.05, ^bP < 0.01 vs before UDCA feeding.

s disease and/or previous ileal resection have elevated bilirubin levels in the bile^[10,14,17,18]. Animal experiments showed that ileectomy-induced bile acid malabsorption increases bilirubin secretion into the bile^[20], suggesting that the increased bilirubin levels in patients with ileal disease and/or resection may be due to induced enterohepatic cycling of bilirubin because of bile acid malabsorption. Orally given ursodeoxycholic acid can compete with ileal absorption of endogenous bile acids and cause bile acid malabsorption in rodents as well as in human subjects^[29-31]. Meéndez-Saánchez *et al*^[21] also showed that oral administration of ursodeoxycholic acid to rodents induces biliary secretion of bilirubin and increases cecal bile acid levels as well as bilirubin concentrations. In the present study however, oral administration of ursodeoxycholic to healthy subjects did not increase the bilirubin concentration in bile, which makes it unlikely that bile acid malabsorption increases bilirubin secretion into the bile in human beings. This is further confirmed by our finding in the present study that patients with Crohn's colitis but without the involvement of the distal ileum and apparent bile acid malabsorption also had elevated bilirubin levels in the bile. In fact, bilirubin concentrations tended to be higher in patients with Crohn's disease confined to the colon than in those with ileal involvement. In contrast to our results, Brink *et al*^[10] and Pereira *et al*^[14] have shown that bilirubin level is normal in the gallbladder bile of patients with Crohn's colitis.

If the increased bilirubin concentration in the bile of patients with Crohn's disease is not due to an enhanced enterohepatic circulation of bilirubin because of bile acid malabsorption, what could then be the explanation? Theoretically increased bilirubin content can be explained by an increased formation and excretion into the bile and/or a decreased metabolism of bilirubin in the intestine with subsequent absorption and enterohepatic circulation of bilirubin. An increased formation of bilirubin may originate from hemolysis. However, none of the patients in the present study had hemolysis or hyperbilirubinemia. Therefore, the most likely explanation for the increased biliary content of bilirubin in the patients is an increased

intestinal absorption. Normally, bilirubin is deconjugated and degraded to urobilinogen and other products in the colon^[32]. In Crohn's disease, an altered colonic bacterial flora may enhance the deconjugation with a subsequently increased absorption of unconjugated bilirubin from the intestine and an increased excretion of bilirubin into the bile^[33].

Two patients with Crohn's disease, one with the disease involving the ileum and the other one with Crohn's colitis were cholecystectomized. The gallbladder bile in both of them was supersaturated with cholesterol and contained cholesterol crystals, indicating that the stones are cholesterol type. Another three patients with ileal involvement but without Crohn's colitis displayed cholesterol crystals in the gallbladder bile. About 1/3 of the patients with Crohn's disease with but without the ileal disease had a short CDT. Half of these patients had unsaturated gallbladder bile. This finding is in agreement with a recent report by Keulemans *et al*^[19] who showed that patients with Crohn's disease have an increased tendency to form cholesterol crystals. They have also found that the crystallization behavior is the same in patients with ileal disease as in those with the disease confined to the colon and is caused by increased cholesterol crystallization promoting activity.

Several of the patients with ileal disease but without gallstones displayed cholesterol saturated gallbladder bile (Table 5). However, the mean value of the cholesterol saturation in this group of patients was the same as that obtained in the healthy subjects, which is in agreement with our previous finding that patients with ileal resection due to Crohn's disease have a normal saturation of the bile^[16,18].

In conclusion, patients with Crohn's disease involving the ileum and those with Crohn's colitis have elevated concentration of bilirubin in the gallbladder bile. Oral administration of ursodeoxycholic acid to healthy subjects does not increase the biliary concentration of bilirubin. These results speak against the previously described hypothesis that the increased concentration of bilirubin in the bile samples from patients with Crohn's ileitis

or previous ileal resection is due to malabsorption of bile acids that spill into the colon where they solubilize unconjugated bilirubin and increase its absorption and enterohepatic circulation. In some patients with ileal disease/resection, the gallbladder bile is supersaturated with cholesterol and contains cholesterol crystals. About 1/3 of the patients with Crohn's disease with but without the ileal involvement have a short CDT probably because of increased cholesterol crystallization promoting activity in the gallbladder bile. Thus, several factors including cholesterol supersaturated bile, short CDT and increased bilirubin concentration, may be of importance for the high risk of developing gallstones of both cholesterol and pigment types in Crohn's disease.

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Crohn's disease in Stockholm County during 1990-2001: An epidemiological update

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Abstract

AIM: To further assess of the incidence and localization of Crohn's disease (CD) in a well-defined population during the 1990s and to evaluate the prevalence of CD on the 1st of January 2002.

METHODS: In a retrospective population based study, all 16-90 years old citizens of Stockholm County diagnosed as having CD according to Lennard Jones' criteria between 1990 and 2001 were included. Case identification was made by using computerized inpatient and outpatient registers. Moreover private gastroenterologists were asked for possible cases. The extent of the disease and the frequency of anorectal fistulae were determined as were the ages at diagnosis. Further, the prevalence of CD on the 1st of January 2002 was assessed.

RESULTS: All the 1 389 patients, 689 men and 700 women, fulfilled the criteria for CD. The mean incidence rate for the whole period was 8.3 per 10⁵ (95%CI 7.9-8.8). There was no difference between the genders. The mean annual incidence of the whole study period for colorectal disease and ileocecal disease, was 4.4 (95%CI 4.0-4.7) and 2.4 (95%CI 2.1-2.6) per 10⁵, respectively. Perianal disease occurred in 13.7% (95%CI 11.9-15.7 %) of the patients. The prevalence of CD was 213 per 100 000 inhabitants.

CONCLUSION: The incidence of CD has markedly increased during the last decade in Stockholm County and 0.2% of the population suffers from CD. The increase is attributed to a further increase of colorectal disease, while the incidence of ileocecal disease has remained stable.

INTRODUCTION

Crohn's disease (CD) is one of the major inflammatory bowel diseases (IBD) that challenges many gastroenterologists in their everyday work. Although CD has been recognized for over 70 years^[1], its etiology still remains unclear. An explanatory factor for the unsolved etiology may be the heterogeneous appearance of the disease.

Although descriptive epidemiological studies can hardly prove the etiology of the disease, they are very important in the pursuit of risk factors that can be further assessed in etiological epidemiological studies. Furthermore, they are useful for planning the health care system, development of new pharmaceuticals and therapeutic endeavors.

The former increasing incidence of CD in countries located in the north^[2-5] seems to have leveled off during the eighties. Nevertheless, the incidence continued to increase in Scotland and Iceland during the eighties and early nineties^[6,7]. An increasing incidence of CD has also been assessed in former low-incidence countries in southern Europe, Asia, and Japan^[8-10]. Although numerous epidemiological studies of CD have been performed since the 1960s, there are only few population-based studies that have explored the incidence of CD during the last decade^[11-14].

When Burrill B Crohn and his colleagues first described CD in 1932^[1], the most common site of the disease was the ileocecal area. It was not until 1960 that CD localized to the colon and/or rectum was regarded as an entity of its own^[15], unrelated to ulcerative colitis (UC). Pure colorectal disease has become more common over the years. In a previous survey of patients diagnosed with CD in Stockholm between 1955 and 1989, the proportion of colorectal disease doubled from 14% to 32% during the study period^[16]. This phenomenon has been reported from other areas as well^[6,7,17].

As the mortality due to CD is low^[18], an increasing

number of patients will suffer from CD. There are scarce current prevalence rates available^[13,19,20].

The primary aim of this study was to further assess the incidence and localization of CD in a well-defined population during the 1990s and to evaluate the prevalence of CD on the 1st of January 2002.

MATERIALS AND METHODS

Study area and population

Stockholm County covers an area of 6 519 km² with both urban and rural parts. The population increased from 1.64 to 1.84 million inhabitants between 1990 and 2001^[21]. The number of individuals aged 16-90 years increased from 1.33 to 1.47 million during the same period and attributed to approximately 80% of the total population. The distribution between the genders was constant during the study period with a share of 49% men. The proportion of aliens was 9.4% in 2001.

There were 9 major hospitals and further 13 established gastroenterologists in private practice taking care of patients with IBD during the study period. The number of lower GI endoscopies (i.e. sigmoidoscopies and colonoscopies) performed in Stockholm County increased from 4 787 to 19 778 per year between 1993 and 2001^[22].

Case identification

In Stockholm County, there has been a computerized central registration of all diagnoses for inpatients since 1969. Since 1993, diagnoses have also been successively registered for outpatients and all patients attending the outpatient clinic at the hospitals. The general practitioners have got their diagnoses registered since 1996. Diagnoses were registered according to the International Statistical Classification of Disease (ICD-9 1987-1996, ICD-10 1997 and onwards).

A survey of possible cases was made from the records registered as CD (ICD code 555 and K.50) in the inpatient register between 1990 and 2002 and the outpatient register between 1993 and 2002.

Gastroenterologists in private practice were asked about possible cases and their colonoscopy and histology reports were assessed. All records (paper, microfilmed, and electronic) were retrospectively scrutinized. Patients with an established probable or definite diagnosis of CD according to Lennard Jones' criteria^[23] were included in the study. Included patients should be citizens of Stockholm County and were diagnosed as having CD between 1st January 1990 and 31st December 2001. Patients younger than 16 years at diagnosis were not included in the study.

Definitions

The diagnosis of CD and extent of the disease at diagnosis were based on radiological and/or endoscopic reports including macroscopic and microscopic findings as previously described in detail^[16].

Date of diagnosis was defined as the first examination revealing signs of CD. If the diagnosis was changed from UC to CD, the first diagnosis date of IBD was considered. Date of presentation of symptoms which are consistent with IBD was approximated to months with a certainty

expressed as either the first or the 15th day of the month. In cases, where only the year could be estimated, the date of onset was set as the 1st of July.

The type of diagnostic examination(s) performed at the time for diagnosis was noted. The localization of CD was determined based on these diagnostic examinations and classified into five groups: oro-jejunal disease, small bowel disease (inflammation of the small bowel excluding the distal 30 cm of the terminal ileum), ileocecal disease (inflammation including the distal 30 cm of the ileum with or without isolated involvement of the cecum), ileocolonic disease (continuous or discontinuous inflammation of the ileum and colon) and colorectal disease (inflammation in the colon only and/or rectum). The group of oro-jejunal disease consisted of patients with solely oro-jejunal disease and patients with mainly oro-jejunal disease but also inflammation in a more distal part of the intestine.

The extent of colorectal disease was further classified into three groups. For assessment of the disease extent, the colon was schematically divided into four areas consisting of the ascending, transverse, descending, and sigmoid respectively. Colonic disease could either be segmental disease with the involvement of one, two or three of the four schematic areas, left-sided disease extending proximally from the rectum but not beyond the splenic flexure, or total colonic disease with inflammation in all the four areas. Patients with segmental or total colonic disease could have either rectal involvement or rectal sparing. Anorectal fistulae included fistulas and abscesses in the rectum, anal canal or perianal area that appeared before the diagnosis of CD, at the time of diagnosis or at any time during the follow-up period.

If data concerning the parameters mentioned above were uncertain to assess, the data were labeled as unreliable and excluded from actual analysis.

Data management and statistical methods

The cases of CD as well as the population were grouped into 15-year age specific groups: 16-30 years, 31-45 years, 46-60, 61-75, and 75-90. Information about the population in Stockholm County was obtained from the National Central Statistical Register^[21].

Incidence rates were calculated for 2-year periods and expressed as annual incidence rates. For each 2-year period, the age specific incidence was calculated by dividing the number of cases by the number of person years in each age group. The population was not adjusted (i.e. subtracted) for previous incidence cases. Calculations were made separately for men and women. The annual incidence was adjusted for sex and age by using the population in Stockholm County in 1995 as a standard population. Incidence rates were expressed as cases per 10⁵ inhabitants with a 95% confidence interval (CI) based on the assumption that the number of cases follows a Poisson distribution. A Poisson model (Breslow) was used to study the relationship between sex, calendar year, age and the risk of CD. The hazard function of individuals aged 16-88 years was assumed to be $\exp[\beta_0 + \beta_1 \cdot \text{minimum}(\text{age}, 45) + \beta_2 \cdot \text{maximum}(0, \text{minimum}(\text{age} - 45, 70 - 45)) + \beta_3 \cdot \text{maximum}(0, \text{age} - 70) + \beta_4 \cdot \text{minimum}(\text{calendar year} - 1990, 6) + \beta_5 \cdot \text{maximum}(0, \text{calendar year} -$

Table 1 New cases of Crohn's disease in Stockholm County during 1990–2001 grouped by 2-year intervals, in relation to gender, age and localization of disease at diagnosis

| | 1990-1991 | 1992-1993 | 1994-1995 | 1996-1997 | 1998-1999 | 2000-2001 | 1990-2001 |
|--------------------------------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| Total | 174 | 219 | 234 | 286 | 235 | 241 | 1 389 |
| Gender | | | | | | | |
| Male | 74 | 114 | 112 | 143 | 127 | 119 | 689 |
| Female | 100 | 105 | 122 | 143 | 108 | 122 | 700 |
| Age at diagnosis | | | | | | | |
| 16-30 | 60 | 86 | 89 | 121 | 87 | 88 | 531 |
| 31-45 | 49 | 52 | 62 | 66 | 57 | 56 | 342 |
| 46-60 | 27 | 44 | 40 | 47 | 51 | 61 | 270 |
| 61-75 | 29 | 27 | 33 | 40 | 32 | 26 | 187 |
| 76-90 | 9 | 10 | 10 | 12 | 8 | 10 | 59 |
| Localization of disease at diagnosis | | | | | | | |
| Oro-jejunal | 2 | 4 | 5 | 7 | 7 | 5 | 30 |
| Small bowel | 8 | 14 | 9 | 9 | 5 | 4 | 49 |
| Ileocecal | 58 | 55 | 61 | 85 | 61 | 73 | 393 |
| Ileocolonic | 15 | 28 | 38 | 35 | 32 | 41 | 189 |
| Colorectal | 91 | 118 | 121 | 150 | 130 | 118 | 728 |

1996)+ $\beta_6 \cdot \text{sex}$]. The coefficients β_1 , β_2 , β_3 , β_4 , β_5 , and β_6 reflected the trends of the risk, $\beta_1 = \beta_2 = \beta_3 = \beta_4 = \beta_5 = \beta_6 = 0$ corresponded to no change of risk^[24].

Time between the onset of symptoms and diagnosis was calculated as the median.

The extent of disease at diagnosis was assessed as incidence rates as well as proportions, i.e. percentages of the total number of cases. Comparison between men and women with respect to the localization of disease was performed by χ^2 test. The Kruskal-Wallis test was applied for comparison of age at onset and calendar time at onset between localization of disease. If a significant importance of localization was obtained, each localization was compared to all the others by use of Fisher's permutation test.

The probability of anorectal fistulae and rectal involvement, depending on age, sex, and localization of the disease, was estimated by the use of logistic regression. The two-tailed tests were used.

The Ethics Committee at Huddinge University Hospital approved the study.

RESULTS

Basic data are shown in Table 1.

Altogether 1 389 patients fulfilled the inclusion criteria. A total of 569 patients (41%) had a definite diagnosis of CD and 820 patients (59%) had a possible diagnosis of CD according to Lennard Jones' criteria.

Information regarding the onset of symptoms, methods of diagnosis, occurrence of anorectal fistulae and rectal involvement was not available in 26, 9, 96, and 31 patients respectively. Twenty-eight percent of the patients were prescribed azathioprine or equivalent immunosuppressive medicine at any time during the follow-up.

Time between onset of symptoms and diagnosis

The median time between the onset of symptoms and

Table 2 Diagnostic procedures performed at the time of diagnosis ($n = 1\,389$)

| | Main examination, n (%) | Total number, n (%) |
|--------------------------|---------------------------|-----------------------|
| Colonoscopy | 690 (49.7) | 690 (49.7) |
| Ileocolonoscopy | 285 (20.5) | 285 (20.5) |
| Large bowel barium enema | 138 (9.9) | 250 (18.0) |
| Enteroclysis | 62 (4.5) | 602 (43.3) |
| Surgery | 82 (5.9) | 141 (10.2) |
| Rigid sigmoidoscopy | 20 (1.4) | NA |
| Sigmoidoscopy | 12 (0.9) | 46 (3.3) |
| Miscellaneous | 45 (3.2) | |
| Unknown | 55 (4.0) | |

NA, not assessed.

diagnosis was 6.5 mo (range 0–376 mo) for the whole study period. Patients with small bowel disease and ileocecal disease had a longer median time to diagnosis (12.8 and 10.9 mo, respectively) than patients with ileocolonic and colorectal disease (6.1 and 5.9 mo, respectively) ($P < 0.005$).

Diagnostic procedures

The diagnostic procedures performed between the first-time visit and the diagnosis of CD are shown in Table 2.

If a colonoscopy/ileocolonoscopy was performed, the examination was considered as the "main examination". Otherwise, the examination that provided most information for diagnosing and evaluation of the extent of disease was labeled as "main examination". The total number of procedures reflected how many patients underwent each specific examination.

Altogether 70.2% of the patients underwent an endoscopic examination of the colon and the terminal ileum was inspected in 29.2% of these patients. An enteroclysis was performed in 43% of all patients. Six percent of the patients were diagnosed by surgery. The remaining patients underwent only flexible/rigid sigmoidoscopy or miscellaneous examinations.

Incidence

The mean annual incidence rate for the entire study period was 8.3% (95%CI 7.9%–8.8%) per 100 000 inhabitants. The mean annual incidence rate was 7.7% (95%CI 7.1–8.3%) and 8.9% (95%CI 8.3–9.6%), respectively, during the first and second half of the study period (Figure 1).

The mean increase in incidence per year was estimated to be 7.6% (95%CI 4.3–11.1%) between 1990 and 1996 with the highest increase (21.0%) between 1990 and 1992 (95%CI 5.4–38.8%) and (14.1%) between 1994 and 1996 (95%CI 1.8–27.8%). The maximum incidence rate was found in 1996–1997 with an annual mean of 10.3% (95%CI 9.1–11.5%) per 100 000 inhabitants. After 1996, the annual incidence decreased with a mean of 4.6% per year (95%CI 1.0–8.0%).

No statistical difference in incidence rates was found between men and women [8.6% (95%CI 7.9–9.2%) *vs* 8.1% (95%CI 7.5–8.7%)] for the entire study period.

Age at diagnosis

The highest age specific incidence was found among those aged 16–30 years at diagnosis with a mean incidence rate

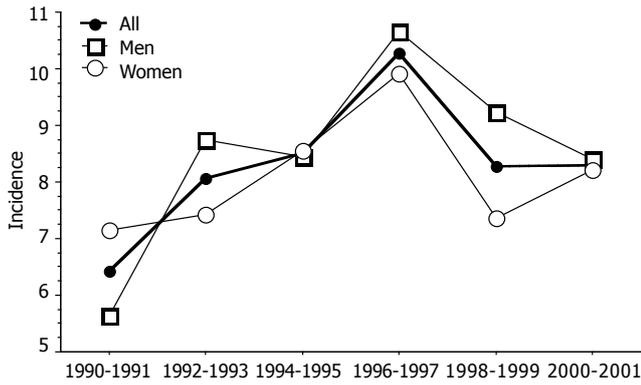


Figure 1 Annual incidence rate (mean per two-year periods $\times 10^{-5}$) of Crohn's disease in Stockholm County (1990-2001).

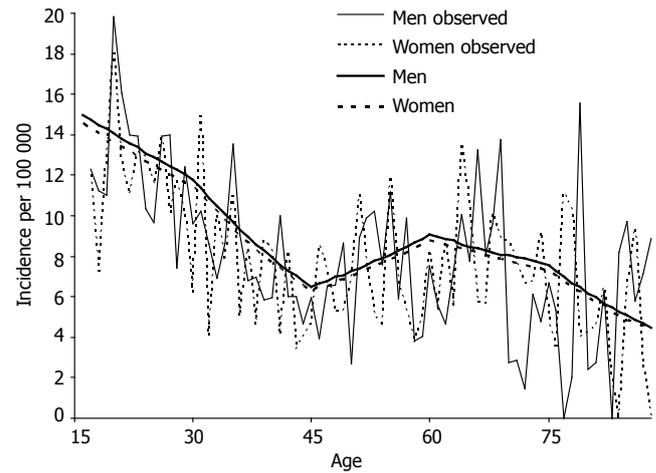


Figure 2 Relationship between age and the incidence of Crohn's disease in Stockholm County (1990-2001), determined by using a poisson model, with the calendar year fixed to 1995, allowing the curve to bend at the arbitrary ages 30, 45, 60, and 75 yr.

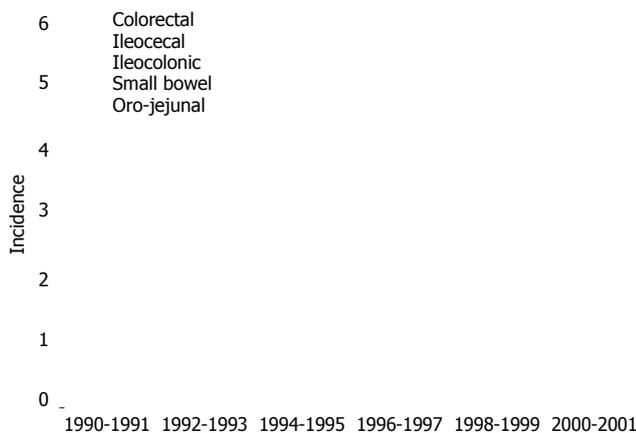


Figure 3 Incidence of Crohn's disease in Stockholm County by the extent of disease at diagnosis per 10^5 inhabitants (1990-2000).

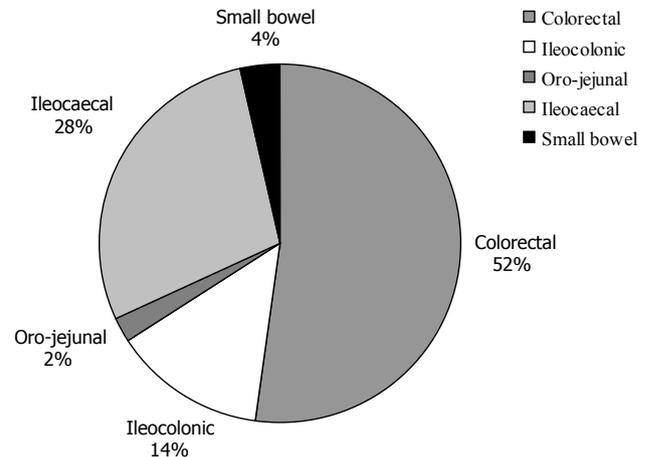


Figure 4 Localization of Crohn's disease in Stockholm County (1990-2001).

of $12.3 \text{ per } 10^5$ (95%CI 10.1-14.6) for the entire study period. A maximum incidence rate was observed during 1996-1997 with $17.1 \text{ per } 10^5$.

The relationship between age and the risk of CD by using a Poisson model, with the calendar year fixed to 1995, allowed the curve to bend at the arbitrary ages 30, 45, 60, and 75 years (Figure 2). With increasing age, the incidence decreased on an average of 1.7% per year between 16 and 30 years ($P < 0.05$) and 3.9% per year between 30 and 45 years ($P < 0.001$). Thereafter, the incidence increased again on a yearly average of 2.2% ($P < 0.05$) resulting in a second peak at the age of 60 years. In the elderly, the incidence rate decreased with an average of 1.2% and 3.9% per year between 60 and 75 years and over 75 years, respectively. Although the crude number of cases was lower in the older age groups, the bimodal peak in incidence was attributed to fewer person-years of follow-up in the older age groups compared with the younger age groups.

The proportion of patients older than 60 years at diagnosis was 17.7% and the proportion of all patients older than 75 years at diagnosis was 4.2%.

Extent of disease at diagnosis

The incidence of CD by the extent of disease at diagnosis is shown in Figure 3.

The mean annual incidence in the whole study period for colorectal and ileocecal disease was 4.4% (95%CI 4.0-4.7%) and 2.4% (95%CI 2.1-2.6%), respectively. Corresponding figures for ileocolonic disease, small bowel disease and oro-jejunal disease were 1.1% (95%CI 1.0-1.3%), 0.3% (95%CI 0.2-0.4%) and 0.2% (95%CI 0.1-0.2%), respectively. The only significant change over time was a decreasing incidence of small bowel disease throughout the study period ($P < 0.05$).

The overall proportion of colorectal and ileocecal disease was 52.3% (95%CI 49.7-55.0%) and 28.3% (95%CI 26.0-30.7%), respectively (Figure 4).

Age influenced the localization of CD at diagnosis. By comparing each disease localization to the others, patients with colorectal ($P < 0.005$) and small bowel disease ($P < 0.005$) were found to be older at diagnosis. In contrary, patients with ileocolonic ($P < 0.001$) and proximal disease ($P = 0.055$) were younger.

In pure colorectal disease, 36.3% of the patients had total colonic involvement, 40.2% had segmental disease and 23.5% had distal colorectal disease. Rectal

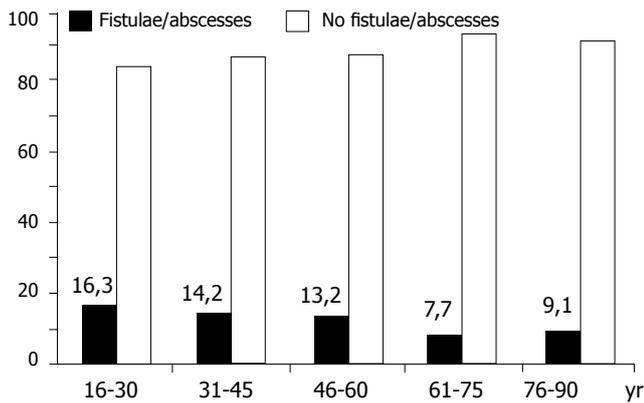


Figure 5 Percentage of patients with Crohn's disease ($n = 1293$) having perianal fistulae/abscesses in Stockholm County during 1990-2001 by age specific groups ($P < 0.05$).

inflammation at diagnosis was found in 67.8% of the patients with colorectal disease.

Distal colorectal disease was associated with a higher age at diagnosis ($P < 0.005$) and inversely total colonic disease was associated with a lower age at diagnosis compared to the rest of the patients ($P < 0.001$). Age did not influence the risk of segmental colorectal disease.

Gender had no influence on either disease localization or extent of colorectal disease at diagnosis.

Anorectal fistulae

Anorectal fistulae occurred before or after the diagnosis of CD in 13.7% (95%CI 11.9-15.7%) of the patients whose data were assessable ($n = 1293$). Gender as well as age had a significant impact on the occurrence of fistulae. Men were more likely to develop this kind of perianal disease than women (15.8% *vs* 11.6%, $P < 0.05$). The impact of age on anorectal fistulae is shown in Figure 5. The disease localization did not influence the frequency of fistulae.

Prevalence

Calculations were based on all adult patients (> 16 years) with CD living in Stockholm County on the 1st of January 2002. Of the 1389 patients diagnosed between 1990 and 2001, 71 patients were dead and 76 patients had moved out from the area. From the earlier cohort of 1936 patients diagnosed during 1955-1989^[16], 368 patients were dead and 239 patients had moved out. Twelve patients diagnosed in Stockholm before 1955 were still living in the area.

The present case identification survey revealed 327 patients diagnosed outside the Stockholm County, 128 patients diagnosed as UC before 1990 and later got the diagnosis changed from UC to CD and 97 patients diagnosed as CD during 1955-1989 but not included in the previous study^[16] for unknown reasons. The prevalence was calculated by dividing the total number of patients with CD (3135) by the adult population (> 16 years) in Stockholm County (1469048). Accordingly, the prevalence of CD among the adults in Stockholm County on the 1st of January 2002 was estimated to be 213 per 100000 inhabitants.

DISCUSSION

Including a former published study^[16], the development of CD has now been documented for 45 years in Stockholm County. Thereby, one of the largest population based cohort of patients with CD in the world has been collected.

There are pros and cons regarding study designs. A prospective study may be considered more faultless but is unfeasible in a large study area with many hospitals and gastroenterologists. Shortcoming of a retrospective study can be lack of uniform diagnostic criteria or incomplete data collection. Patients in Stockholm are handled by a limited number of gastroenterologists and the diagnosis is therefore uniform. However, endoscopy has been escalating during the last years. A potential aim to retrospectively assess smoking habits in this study was abandoned due to the lack of reliable data, else sufficient data regarding different parameters were available. The pro of the present retrospective study is a long follow-up period when case ascertainment can be accomplished in the meantime.

The number of patients having a definite diagnosis of CD according to Lennard Jones' criteria^[23] in this study was lower (41%) than that in our previous study of CD in Stockholm (73%)^[16]. One explanation could be that fewer patients underwent surgery. Specimens for histopathological examination were less available and thereby granulomas and submucosal signs of CD might be undetected. Some uncertainty may arise about the "probable" cases of CD according to Lennard Jones' criteria. Case ascertainment was assured by scrutinizing the records retrospectively. By following the clinical course of CD, the diagnosis was further ascertained and all included cases were bonafide cases of CD.

Long-term incidence data are scarce in the literature from the late 1990s and onwards^[12-14,25-27]. The incidence of CD among the adults in the present study was 70% higher than that in Stockholm during 1985-1989, with the highest incidence rate in 1996-1997. The registry of outpatients begun in 1993 and theoretically, the lower incidence found in 1990-1993 may represent an incomplete case ascertainment. Similarly, the drop-off in incidence during the last 4 years of the study could represent decreased ascertainment. However, all patients with CD in Stockholm are regularly followed up and should be identified sooner or later. Diagnoses are registered continuously and a marked delay of identification is unlikely, thus the case ascertainment is reliable.

A 55% increase of the incidence rate was also seen in Denmark where the incidence rate reached a maximum with approximately 10 per 10⁵ in 1998-2002^[12]. In Northern France, the incidence of CD increased 23% during a similar time period^[14]. Still, the overall incidence rate is lower in France than in Stockholm. The highest incidence rates have previously been reported from North Eastern Scotland^[7] and Manitoba in Canada^[19] and now also from Sweden. This may illustrate a north-south gradient of CD that still exists although less pronounced than believed earlier^[10]. The proportion of immigrants in Stockholm has not changed during the last decade and

should therefore not affect the results^[21].

CD is in general believed to affect women more frequently than men^[14,17,28] although several studies have not found any difference^[4,10]. This study confirmed earlier observations from Stockholm that gender does not influence the risk for CD^[16].

CD mostly occurs in young adults, but can present itself at any time in life^[29]. The age specific distribution in this study is consistent with some former studies with the highest incidence found among young adults and thereafter decreasing incidence rates with increasing age and a second peak in the elderly^[7,16,17,30]. Nevertheless, the overall incidence among the elderly was substantially higher in this study compared with other studies^[31,32].

Pure colorectal disease and small bowel disease are associated with a high age in contrast to our previous study where only small bowel disease was related to a high age at diagnosis. It has been suggested that smoking CD patients are more likely to develop small bowel disease than non-smoking patients^[33]. Smoking habits were not evaluated in this retrospective study. However, one can speculate whether smoking was more tolerable in the society previously with an ensuing larger group of smokers among the older population. This assumption is strengthened by the fact that this entity of CD was the only one that decreased throughout the study period. Colorectal CD may be misclassified as ischemic colitis as well as diverticulosis. Both conditions are common in this age group. CD patients with concomitant diverticular disease are more likely to have granulomas but the misclassification should be limited by finding granulomas in other parts of the intestine^[31]. Focal inflammation is one of the criteria of CD and distinguishes colorectal CD from UC.

This study focused on CD in adults since a study of children with IBD in northern Stockholm has been recently performed^[27]. That study showed an increasing incidence of pediatric CD during the latter 1990s with an incidence of 8.4 per 10⁵ in 1999-2001, thus identical rates were found in adults in the present study.

Anorectal fistulae, strongly associated with CD, have been reported in almost 40% of the patients, preferably in those with colorectal involvement^[34]. Though patients with colorectal disease accounted for a huge proportion, the frequency of anorectal fistulae was low in the present study and on the whole, no relation was found between the frequency of fistulae and the disease localization. Inclusion of patients with mild colorectal disease might cause a dilution effect and a false low frequency of fistulae. A short follow-up period may be an alternative explanation and moreover, a general increased use of azathioprine may theoretically have prevented the development of fistulae.

The increasing incidence of CD is found in patients with solely colorectal disease. This entity of CD was first described in 1960 but an increasing proportion of colorectal CD was not reported before the late eighties^[15].

The increase of colorectal disease can be attributed to a factual increase, a switch from UC to CD or more accurate diagnostic procedures. Unfortunately, there are no contemporary data regarding the development of UC in Stockholm County. However, Jacobsen *et al*^[12] have shown a concomitant substantial increase of both UC and CD

in North Jutland in Denmark between 1988 and 2002, which supports a factual increase of CD and contradicts a diagnostic shift from UC to CD. In contrast, the incidence of UC decreased by 17% in Northern France during a comparable time period^[14]. If hypothetically, the decreasing incidence of UC should represent a corresponding increase of CD, still there would be a 50% increase of CD not due to a reclassification from UC to CD in Stockholm during the 1990s. The diagnosis of CD is partly based on histopathology and may vary between different pathologists^[35]. In this study one single pathologist did most of the histopathological examinations.

The number of lower GI endoscopies performed in the area has increased fourfold^[22]. This escalating access to medical service may have led to an examination of more patients with only mild symptoms. Patients with minor colonic inflammation that might have been overlooked with previously used barium enemas could therefore have attributed to the increased incidence of CD.

The median time between presentation of symptoms and diagnosis is rather similar to that found in 1955-1989^[16]. The shortest time is found in patients with disease in the colon, while patients with small bowel disease have a longer period of symptoms before the diagnosis. This fact may illustrate the advantage of endoscopic technique in the diagnosis of IBD. Recently, the capsule endoscopy has been introduced in Sweden and is used to a limited extent^[36]. With a more widespread use of this new technique, disease in the ileum may be more frequently detected^[37]. Consequently an increased number of patients with small bowel CD will be diagnosed and thereby the incidence of CD will probably further increase in the future.

In conclusion, this follow-up study of CD in Stockholm County shows that the incidence increases by 80% from the 1980s to the 1990s. As much as 0.2% of the adult population in Stockholm County suffers from CD. The disease appears at any age and there is no predominance regarding gender. Colorectal CD is the most common localization and the frequency of anorectal fistulae is less evident than that in the past.

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CLINICAL RESEARCH

Proton pump inhibitor treatment of patients with gastroesophageal reflux-related chronic cough: A comparison between two different daily doses of lansoprazole

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Abstract

AIM: To compare two different daily doses of lansoprazole given for 12 weeks and to assess the role of gastrointestinal (GI) investigations as criteria for selecting patients.

METHODS: Out of 45 patients referred for unexplained chronic persistent cough, 36 had at least one of the GI investigations (endoscopy, 24-h esophageal pH-metry and a 4-week trial of proton pump inhibitor (PPI) therapy) positive and were randomly assigned to receive either 30 mg lansoprazole o.d. or 30 mg lansoprazole b.i.d. for 12 weeks. Symptoms were evaluated at baseline (visit 1) after the PPI test (visit 2) and after the 12-week lansoprazole treatment period (visit 3).

RESULTS: Thirty-five patients completed the study protocol. Twenty-one patients (60.0%) reported complete relief from their cough with no difference between the two treatment groups (58.8% and 61.1% had no cough in 30 mg lansoprazole and 60 mg lansoprazole groups, respectively). More than 80% of the patients who had complete relief from their cough at the end of the treatment showed a positive response to the PPI test.

CONCLUSION: Twelve weeks of lansoprazole treatment even at a standard daily dose, is effective in patients with chronic persistent cough. A positive response to an initial PPI test seems to be the best criterion for selecting patients who respond to therapy.

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Key words: Gastroesophageal reflux; Cough; Proton pump inhibitors; Lansoprazole

INTRODUCTION

There is increasing evidence that many otolaryngologic or pulmonary conditions, ranging from very mild symptoms such as hoarseness to very severe diseases such as cancer, may be caused by gastroesophageal reflux (GER)^[1]. In particular, GER seems to account for a relevant proportion of patients with asthma, cough, and laryngitis. Chronic persistent cough is a very common and disabling complaint for which patients seek medical care. It has been shown that in non-smoking patients with a normal chest X-ray and not taking angiotensin-converting enzyme (ACE) inhibitors the four most common causes of cough are post-nasal drip syndrome (PNDS), asthma, GER and chronic bronchitis^[2]. These four conditions may account for as many as 90% of the cases of chronic cough. Moreover, GER by itself has been found to be responsible for 10%-40% of them even in the absence of reflux symptoms^[3]. For these reasons, antisecretory drugs have been proposed for the treatment of patients with chronic cough possibly due to GER. Most studies on this topic have been performed with proton pump inhibitors (PPIs) at higher daily doses and for generally longer periods than those usually employed for typical GER disease. These studies have shown better results in terms of symptom resolution and improvement of laryngeal signs for PPIs vs placebo, but the success rate has been relatively low and quite variable, ranging from 35% to 60%^[4-6]. This variability in response may be due to the differences either in selection criteria or in treatment regimens. Since the poorest outcomes^[4] have been obtained in the study in which PPIs were given at the highest daily dose and for the longest period, patient selection may be the critical factor.

Thus, published studies suggest that antireflux treatment of patients with GER-related respiratory symptoms, particularly chronic cough, must be carried

out with profound and prolonged acid inhibition in order to achieve satisfactory results in about two-thirds of the cases. However, the optimal treatment regimen and the criteria for patient selection need to be better defined.

The aim of our study was to evaluate the relative efficacy of two different daily doses of lansoprazole given for 12 weeks to patients with chronic persistent cough that could be reasonably ascribed to GER, and to assess the role of gastrointestinal (GI) investigations (endoscopy, 24-h esophageal pH-metry and a 4-week trial of empiric PPI treatment) as criteria for selecting patients with chronic cough who could benefit from antireflux treatment.

MATERIALS AND METHODS

Patients

The study protocol was approved by the Ethics Committee of the S. Orsola-Malpighi Hospital, Bologna, Italy and all participants gave their written informed consent. A pretrial analysis determined that 15 patients were required in each treatment to demonstrate equivalence between 30 and 60 mg/d lansoprazole treatment groups in terms of cough severity and frequency with 80% power at an alpha level of 0.05.

Patients aged 18-70 years with unexplained chronic persistent cough (i.e. for at least 3 days per week for a minimum of 3 months) were enrolled in the study over a period of 1 year (from June 2002 to June 2003). All patients were consecutively referred by otolaryngologists and pulmonologists after the exclusion of oropharyngeal or respiratory diseases potentially responsible for the cough (particularly asthma and PNDS) by means of a diagnostic evaluation which included medical history, physical examination, methacholine challenge test, chest X-ray and fiber-optic laryngoscopy. Patients were excluded if they were pregnant or breastfeeding; had systemic diseases, cardiac and pulmonary disorders, viral and bacterial or fungal infections, neoplasia or Zollinger–Ellison syndrome; or received previous treatment with drugs that interfered with their gastric acid secretion (H₂-antagonists, PPIs) and chronic treatment with NSAIDs, phenytoin, warfarin, tricyclic antidepressants, reserpine, beta-agonists, anticholinergics, antihistamines, inhaled steroids, or ACE inhibitors. Patients with chronic alcohol or drug abuse were also excluded, as were smokers.

After an initial clinical evaluation aimed at assessing the presence of associated typical reflux symptoms (heartburn and regurgitation) and the severity of cough, all patients underwent a diagnostic work-up which included upper GI endoscopy, 24 h esophageal pH-metry and a trial of empiric PPI therapy (PPI test, 30 mg lansoprazole b.i.d. for 4 weeks). The investigations were always performed in the same sequence, i.e. in the order of endoscopy, pH-metry and the PPI test.

A patient was considered eligible for 12 wk of lansoprazole treatment if at least one of the GI investigations was positive.

Symptom assessment

The severity of cough was evaluated according to a visual analog scale (VAS) graded from 0 to 10 and to a four-level

scoring system, regarding the previous week, calculated as follows:

- *Overall frequency*: 0 = absent, 1 = occasional (<3 d/wk), 2 = often (3-6 d/wk), 3 = every day
- *Daily frequency*: 0 = absent, 1 = 1 episode, 2 = 2-3 episodes, 3 = >3 episodes
- *Severity*: 0 = absent, 1 = mild (not interfering with daily activities), 2 = moderate (sometimes interfering with daily activities), 3 = severe (regularly interfering with daily activities and/or sleep).

Symptoms were evaluated at baseline (visit 1), after a 4-wk PPI trial (visit 2) and a 12-week lansoprazole treatment period (visit 3).

Upper GI endoscopy

All patients underwent upper GI endoscopy performed by the same gastroenterologist. The presence of esophagitis was noted and graded according to the Savary-Miller classification: grade I = single erosive or exudative lesion, oval or linear, on only one longitudinal fold; grade II = non-circular multiple erosions or exudative lesions on more than one longitudinal fold with or without confluence; grade III = circular erosive or exudative lesions; grade IV = chronic lesions, ulcers, strictures, or short esophagus, isolated or associated with grade I-III lesions; grade V = Barrett's epithelium, isolated or associated with grade I-III lesions^[7]. Grades I-IV were considered diagnostic of erosive esophagitis and endoscopy was considered positive for the purpose of this study.

Twenty-four h esophageal pH-metry

After an overnight fast, two glass electrodes previously calibrated with buffer solutions at pH 7.0 and 1.0, were assembled with the sensors 15 cm apart and introduced into the stomach by the nasopharyngeal route. The distal electrode was positioned 5 cm above the lower esophageal sphincter (LES) so that the proximal one was located distal to the upper esophageal sphincter (UES). The location of the LES was determined by a combination of pH step-up technique, manometry (always performed in patients with hiatus hernia; 8 cases) and visualization under fluoroscopy if needed. The probe was connected to a digital portable recording unit (pH-day, Menfis, Italy) with a sampling frequency of one signal every second. Patients were asked to maintain their usual lifestyle and diet. They were provided with a diary card to record timing of meals, duration of nocturnal rest, time of cough occurrence and type of symptoms. Moreover, they were instructed to push an event marker button on the recording unit at the time of occurrence of their major complaint (cough). The pH-metry was stopped after 23-24 h. After being extracted, the electrodes were calibrated again with buffer solutions at pH 7.0 and 1.0. GER was evaluated as acid exposure fraction time (percent of time with pH <4). For the purpose of this study, pH-metry was considered positive if the percentage of total time with pH <4 was >4.73%^[8] at the distal recording site or >1.00% at the proximal site (these values exceeded the 95th percentile of those previously obtained in normal subjects in our laboratory). In patients with normal values at both

Table 1 Demographics, clinical details and outcome of investigations performed in the 45 patients with chronic cough

| | All patients | GERD symptoms | |
|------------------------------|--------------|---------------|------------|
| | | Present | Absent |
| Subjects | 45 | 25 | 20 |
| Age (mean±SD) | 54.5 ± 11.1 | 54.6 ± 12.8 | 54.4 ± 8.6 |
| Gender, male (%) | 7 (15.5) | 3 (12.0) | 4 (20.0) |
| Upper endoscopy positive (%) | 7 (15.5) | 3 (12.0) | 4 (20.0) |
| pH-metry positive (%) | 26 (57.8) | 13 (52.0) | 13 (65.0) |
| PPI test positive (%) | 23 (51.1) | 13 (52.0) | 10 (50.0) |

No statistical differences between the groups in any of the parameters.

recording sites, we calculated the symptom index (SI), i.e., the number of cough episodes associated with acid reflux (that was simultaneous with or occurring within 5 min before or after the reflux episode) divided by the total number of cough episodes multiplied by 100, together with the symptom sensitivity index (SSI), i.e., the number of cough episodes associated with acid reflux, divided by the total number of reflux episodes multiplied by 100. We considered the investigation positive, if the SI was >50% with a SSI >20%^[9].

PPI test

After endoscopy and 24-h pH-metry, all patients received a 4-week open-label course of 30 mg lansoprazole administered before breakfast and dinner. Before PPI trial (visit 1) was started and at the end of the treatment course (visit 2) the patients were interviewed regarding their main symptom, i.e., cough, and asked to rate symptom severity during the previous week on a VAS graded 0-10^[10]. The test was considered positive, if the post-treatment value was 0 or if the difference between the pre- and post-treatment values was ≥5.

Intervention

Patients with a positive finding in at least one of the three assessments (endoscopy, pH-metry and symptom relief during a 4-week PPI trial) were randomized to either 30 mg lansoprazole oral capsules in the morning and identical placebo capsules in the evening or 30 mg lansoprazole in the morning and 30 mg lansoprazole in the evening before meals for 12 wk. Active drug and placebo were both supplied by Takeda Italia Pharmaceuticals (Rome, Italy). Both patients and investigators were blinded to the treatment status. Patients were interviewed and VAS was assessed at the end of treatment, and pill counting was performed to check for compliance with treatment.

Symptomatic response was defined as complete (VAS and score values = 0), partial (VAS value ≥ 1 but ≤ 50% of the baseline value and score value <5) or absent.

Statistical analysis

Data were analyzed by the Student's *t* test for independent data of continuous variables, by the Mann-Whitney's non-parametric test for discrete variables and by the χ^2 test for nominal variables reported as contingency tables^[11]. The Wilcoxon's rank-sum test or the Friedman test was

Table 2 Analysis of concordance between 24 h pH-metry and PPI test

| | 24 h pH-metry | | Total |
|-------------------|---------------|----------|-------|
| | Positive | Negative | |
| PPI test positive | 12 | 11 | 23 |
| PPI test negative | 14 | 8 | 22 |
| Total | 26 | 19 | |

Youden's index = -0.1174 K (measure of reliability) = -0.115 (no reliability).

used for the comparison of symptom scores. A two-sided significance level of 0.05 was chosen.

Equivalence between the two treatment regimens was determined using the Schuirman one-sided test procedure. Mean VAS values recorded at visit 3 (after 12 wk of double-blind therapy) in the reference group (60 mg/day lansoprazole) were transformed to 100, and then the same transformation equation was applied to the mean VAS values at visit 3 in the 30 mg/d lansoprazole group. Equivalence was demonstrated if the 90% confidence intervals (CIs) for the difference between the transformed mean VAS values in each group were within the standard deviation (± 20).

RESULTS

Patient selection

Forty-five patients (7 men, mean age 54.5 ± 11.1 years, age range 29-70 years) referred for unexplained chronic persistent cough underwent the GI investigations aimed at the detection of GER disease (Table 1). Most of the patients were middle-aged females and 25 (55.5%) also reported typical reflux symptoms (heartburn and/or regurgitation occurring at least once per week). Thirty-six (80%) patients were positive for at least one test. Only a few patients ($n = 7$, 15.5%) had endoscopy-proven esophagitis, while about 50% had pathological GER and a positive PPI test. Twenty-four patients (53.3%) were found to have pathological reflux, 12 at both recording sites, 10 at the distal site only and just 2 at the proximal site only. Two out of 21 patients with negative pH-metry were found to have a positive SI and considered as positive for pH-metry. Twenty-three patients had a positive PPI test, five of them reported complete disappearance of cough, while the remaining 18 had a reduction in cough symptoms of 76.8% ± 10.1%. There was no association between an abnormal result of any of the aforementioned tests and the presence or absence of associated GER symptoms. There was a lack of concordance between the results of pH-metry and those of the PPI test (Table 2). In fact, 12 patients were positive for both tests and 8 were negative for both tests (44.4%), while in the other 25 cases the tests provided opposite results.

Of the 36 patients who satisfied the criteria for admission to the 12-week lansoprazole treatment, 12 had positive PPI test and pH-metry, 11 had a positive PPI test only and 13 had positive pH-metry only. Esophagitis was not the only pathological finding in any of the patients. One of the patients did not continue the study due to

Table 3 Characteristics of the patients entering the randomized double-blind phase of the study

| | Lansoprazole (30 mg/d) (n = 17) | Lansoprazole (60 mg/d) (n = 18) |
|------------------------------|------------------------------------|------------------------------------|
| Age (mean±SD) | 57.5 ± 11.9 | 52.4 ± 10.0 |
| Gender, male (%) | 3 (17.6) | 2 (11.1) |
| Upper endoscopy positive (%) | 4 (23.5) | 3 (16.7) |
| pH-metry positive (%) | 13 (76.5) | 10 (55.5) |
| PPI test positive (%) | 11 (64.7) | 11 (61.1) |

No statistical differences between the groups in any of the parameters.

Table 4 Patient symptoms evaluated at baseline (visit 1), after the PPI test (visit 2) and at the end of the 12-week lansoprazole treatment period (visit 3)

| | Visit 1 | | Visit 2 | | Visit 3 | |
|------------------------------------|-------------|---------|--------------------------|------------------------|------------------------|-------------------------|
| | VAS | Score | VAS | Score | VAS | Score |
| Lansoprazole (30 mg/d) (n = 17) | 8 (7.5-9.5) | 9 (8-9) | 2 (1.5-5.5) ^b | 4 (3-7) ^a | 1 (0-4.5) ^b | 3 (0-6.5) ^b |
| Lansoprazole (60 mg/d) (n = 18) | 9 (8-9) | 8 (7-9) | 2 (1-6.5) ^b | 3.5 (3-7) ^a | 1 (0-5) ^b | 3 (0-6.25) ^b |

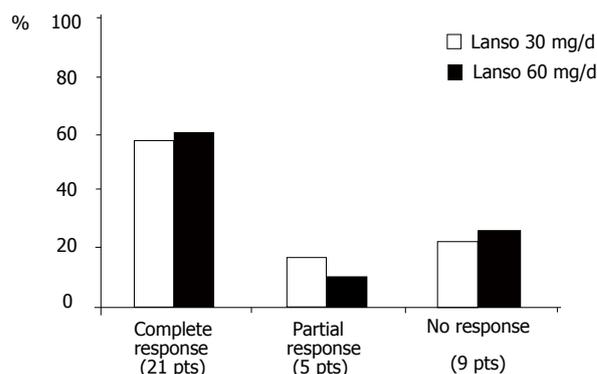
Data expressed as median (25%-75% quartiles). ^bP < 0.001 vs visit 1; ^aP < 0.005 vs visit 1.

unwillingness, while the remaining 35 completed the study protocol (17 on 30 mg/d lansoprazole and 18 on 60 mg/d lansoprazole) and were considered for our efficacy analysis. There was no statistically significant difference between the two treatment groups with regard to age, gender, presence of esophagitis, or 24-h pH-metry and PPI trial outcome (Table 3).

Effect of treatment

Patients' symptoms as assessed with both the subjective (VAS) and the objective (score) system, improved significantly at the end of the 12-wk treatment period vs baseline in both treatment groups, with no statistically significant difference either between the two groups or between the values at visits 2 and 3 (Table 4). The 90% CIs of the difference in the transformed mean VAS scores at 12 wk were within the standard deviation, thereby demonstrating equivalence of the two regimens.

At the end of the 12-wk treatment period 21 patients (60.0%) reported complete relief of their cough with no difference between the two treatment groups (10/17 and 11/18 had no cough in the 30 mg/d lansoprazole and 60 mg/d lansoprazole groups, respectively, Figure 1). In patients selected for the 12-wk course of lansoprazole a PPI was actually taken for 16 wk (including the 4-wk PPI test). At the end of the PPI test, 10 (28.6%) of the 35 patients who continued to complete 12 wk of therapy showed complete relief of their cough. During the following course of PPI the percentage of symptom-free patients increased up to about 60% at the end of the 3-mo period, independently of the daily dose of the drug.

**Figure 1** Percentage of patients showing symptomatic response at the end of 12-wk treatment with 30 mg/d (n = 17) or 60 mg/d (n = 18) lansoprazole.

Patient characteristics and outcome of treatment

In order to determine the factors that may have influenced the treatment outcome, we compared the characteristics of patients who achieved complete relief of their symptoms with those who showed only partial relief or no symptomatic response (Table 5). As shown in the table, the positivity of the PPI test was significantly different between the two groups. In fact, more than 80% of patients who had complete relief of their cough at the end of treatment had a positive response to the PPI trial in comparison to only 28.6% of those who did not satisfactorily respond. None of the other factors, including the daily lansoprazole dose, was significantly correlated with the positive outcome of the treatment. Moreover, the rate of complete symptom relief at the end of the 12-wk course in patients classified as PPI test-positive was 81.8% in comparison to only 23.1% in those with a negative test (Figure 2).

DISCUSSION

The correlation between respiratory or otolaryngologic symptoms and GER is usually ascertained in patients with unexplained chronic cough by the exclusion of other common causes such as asthma and PNDS, and the finding of pathognomonic signs such as posterior laryngitis, also called "reflux laryngitis", as well as the demonstration of pathological reflux^[4,6].

In our study, the pathogenetic role of reflux was suspected after the exclusion of common otolaryngologic or pneumological causes and after a careful work-up aimed at the detection of GER disease. The presence of posterior laryngitis was not included in our criteria because the sensitivity of this finding is generally thought to be low, while its specificity is ill-defined^[12]. About 50% of our patients complained of typical reflux symptoms, such as heartburn and regurgitation, but this finding seemed to be of little relevance, since it was not correlated with the presence of esophagitis, pathological reflux or specifically, a positive response to the PPI trial (Table 1).

As expected, only a few patients had endoscopically proven esophagitis. This is in agreement with the previous observations in patients with so-called extra-esophageal manifestations of GER disease, showing that

Table 5 Comparison between patients with symptomatic response and non-responders

| | Responders [n = 21 (%)] | Non-responders [n = 14 (%)] |
|-------------------------------------|-------------------------|-----------------------------|
| GER symptoms | 14 (66.7) | 5 (35.7) |
| Treatment with 60 mg/d lansoprazole | 11 (52.4) | 7 (50.0) |
| Erosive esophagitis | 5 (23.8) | 2 (14.3) |
| pH-metry positive | 10 (47.6) | 13 (92.8) |
| PPI test positive | 18 (85.7) | 4 (28.6) ^b |

Responders = patients with VAS and score values of 0; non-responders = patients with VAS and score values ≥ 1 ; ^b $P < 0.01$ vs responders.

endoscopic evidence of esophagitis is found in 25%-40% of patients with asthma^[13-14] and in only 10%-25% of patients with otolaryngologic disorders^[6,15]. Therefore, upper GI endoscopy has a very low sensitivity in patients with atypical GER symptoms and, in the absence of other clinical indications, should not be included in the diagnostic work-up of these patients.

Ambulatory 24-h esophageal pH-metry is considered the gold standard for GER detection, with a high sensitivity in patients with typical symptoms^[16]. Studies performed in patients with extra-esophageal symptoms showed that diagnostic sensitivity ranges 50%-80% in patients with asthma^[17,18] and otolaryngologic manifestations, such as laryngitis or cough^[4,6,19]. Moreover, the use of a double esophageal probe has been proposed in the latter patient group^[19]. However, the usefulness of proximal pH-metry has been questioned since its sensitivity and reproducibility are too low^[14,20]. Our results have confirmed the findings of others, showing that the diagnostic gain provided by dual-channel pH-metry is very low^[4].

Since the presence of "physiological" reflux does not necessarily imply the absence of a casual link between reflux and symptoms, the diagnostic sensitivity of 24-h pH-metry may be improved by including the evaluation of a symptom/reflux correlation (SI), especially in patients with atypical symptoms, such as non-cardiac chest pain^[21]. On the other hand, the SI has two major potential limitations: (1) it is feasible only in patients with symptoms during the recording period and this is not always the case (in our patient group 77.7% reported cough episodes during pH-metry), and (2) its value may be questioned if patients have only few symptomatic episodes during the recording period. For this reason, as suggested by previous studies^[22], we considered a correlation between a given symptom and a reflux event to be present for SI values $> 50\%$ together with a SSI $> 20\%$. We performed this evaluation as part of the analysis of our 24 h pH-metry tracings and found that 17 of the 21 patients with negative pH-metry reported cough during the recording period, with 2 of them showing a positive SI, thus increasing the diagnostic value of this investigation.

An empirical trial with PPIs (the so called "omeprazole or PPI test") has been recently proposed for the diagnosis of GER disease in patients with various clinical presentations, especially those with atypical or extra-esophageal symptoms. The diagnostic sensitivity

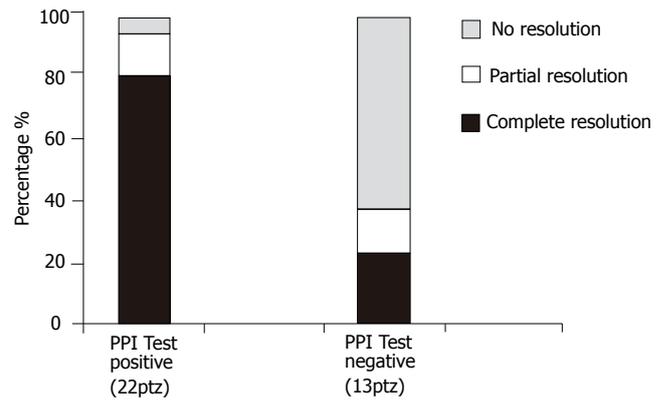


Figure 2 Percentage of patients showing symptomatic response at the end of 12-wk treatment with 30 or 60 mg/d lansoprazole ($n = 35$), subdivided according to the outcome of PPI test. $P < 0.05$ vs negative PPI test.

of this test is generally validated vs 24-h pH-metry as a gold standard. The variability of results reported thus far depends on the differences in treatment schedules (daily doses, duration) and the outcomes chosen (cut-off point for symptom improvement, VAS, questionnaires, etc.). Doses used in clinical studies range 40-80 mg of omeprazole or 60 mg of lansoprazole daily from 1 to 14 d in patients with symptoms suggestive of GER disease or non-cardiac chest pain^[23-29] and from 7 to 90 d in patients with extra-esophageal manifestations^[4,30,31]. In patients with laryngeal symptoms, in which a longer duration and a high daily dosage are considered necessary for obtaining a significant symptom improvement, the test is in general fairly sensitive, with values ranging 62.5%-81%^[4,30,31]. Thus, our PPI test consisted of 4-week administration of 60 mg/d lansoprazole. According to a similar study^[28] we used a VAS graded 0-10 to assess as objectively as possible the severity of symptoms perceived by our patients and we considered the PPI test positive if symptoms disappeared (grade 0) or were reduced by at least 50% in comparison to the pre-administration value. In our study the test was positive in 63.9% of patients with a sensitivity similar to that previously reported.

In our study there was a lack of concordance between the results of the 24 h esophageal pH-metry and those of the PPI test (Table 2). In fact there was agreement in only 20 patients (44.4%). It was reported that there is a significant direct correlation between pH-metry and response to the omeprazole test in patients with typical reflux symptoms (65.8% of patients with concordant results, $P = 0.04$)^[23]. In contrast, a study^[4] performed in patients with chronic cough showed concordance between the two tests in only half the cases (52.2%). This lack of concordance is not surprising and may depend on the intrinsic limitations of both pH-metry (i.e. absence of symptoms during the recording period in patients with "physiological" reflux) and the PPI test (inadequacy of treatment dose or duration). It may also depend on the simultaneous and independent existence of two conditions, GER and cough, not causally linked.

For all these reasons in our study, we proposed that at least one of the GI investigations (endoscopy, pH-metry,

PPI test) had to be positive in order to select the cough patients in whom a 12-wk lansoprazole course could be reasonably proposed as a treatment choice.

The efficacy of PPI treatment in patients with extra-esophageal manifestations has been previously established^[4,6]. Our study showed that both lansoprazole regimens significantly reduced symptom scores in comparison to baseline values (Table 4). There was a further reduction of the values recorded at visit 3 in comparison to those at visit 2, but the difference was not statistically significant. Moreover, it is interesting to note that there was a good concordance between the subjective (VAS) and objective (score) analysis, suggesting that they can be used interchangeably for symptom assessment. The success rate was also evaluated on an individual basis in terms of symptom resolution (Figure 1) which was graded on three levels according to the complete, partial or poor improvement of the symptoms. At the end of the 12-week treatment course about 60% of the patients reported complete relief of their cough (58.8% and 61.1% had no cough in the 30 mg/d lansoprazole and 60 mg/d lansoprazole groups, respectively). We did not find significant differences between the two treatment regimens but our study was only powered to show equivalence rather than efficacy. Studies performed in patients with chronic cough treated with PPIs have reported a complete relief of the symptom in a percentage of patients ranging 26%-43%^[4,5] but the comparison among the studies is difficult because of different patient selection criteria and treatment regimens. A study that used the same drug and treatment duration as the present study^[6] has reported a complete resolution of symptoms in 50% of patients with idiopathic laryngitis, which is very similar to our results.

In our study, the patients selected for the 12-wk course of PPI after the test period actually took lansoprazole for 16 weeks, and some of these patients experienced symptom relief at the end of the first 4 weeks, 10 of them showing complete resolution of their cough. This suggests that 1-month PPI administration at a double daily dose may be an effective therapy in about one-third of these cases. The percentage of symptom-free patients increased up to about 60% at the end of the 12-wk period, independently of the daily dose of the drug.

The analysis of factors that could have influenced the outcome (Table 5) clearly showed that the positivity of the PPI test was significantly different between complete and incomplete responders to PPI treatment. None of the other factors, particularly the presence of GER symptoms, seemed to be associated with the positive outcome of treatment. This is in agreement with previous studies in patients with cough^[4,6] and laryngitis^[6]. Indeed, the response to an initial treatment with a PPI has been suggested as the best method for identifying patients with GER-related chronic cough^[4]. Our results show that PPI test may predict response to a longer course of PPI treatment in patients with atypical reflux symptoms. The rate of complete symptom relief at the end of the 12-week course in our patients classified as PPI test-positive was 81.8% (the highest, to our knowledge, among those reported in similar studies) in comparison to only 23.1% of those with a negative test (Figure 2).

In conclusion, a 12-wk course of lansoprazole is effective in relieving symptoms of patients with unexplained chronic persistent cough. A positive response to an initial 4-wk administration of PPI at a double daily dose seems to be an effective criterion for selecting patients who obtain the best results from the PPI treatment.

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Benefits of early postoperative jejunal feeding in patients undergoing duodenohepaticoduodenectomy

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opportunity for patients who have undergone DHP for a peri-ampullary mass.

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Abstract

AIM: To study whether early postoperative enteral nutrition reduces the incidence of complications and/or improves nutritional status following duodenohepaticoduodenectomy (DHP).

METHODS: We studied 39 patients who underwent DHP for a peri-ampullary mass. Twenty-three patients received total parental nutrition and then started to have an oral intake of nutrition between postoperative day (POD) 7 and 14 [late postoperative enteral nutrition (LPEN) group]. Sixteen patients started to have enteral feeding through a jejunostomy catheter the day after the operation [early postoperative enteral nutrition (EPEN) group]. The incidence of complications and laboratory data at the early postoperative stage were studied in comparison between LPEN and EPEN groups.

RESULTS: Serum levels of albumin and total protein in the EPEN group were significantly higher than those in the LPEN group. The loss of body mass index was significantly suppressed in the EPEN group as compared to the LPEN group. The lymphocyte count decreased immediately after the operation was restored significantly faster in the EPEN group than in the LPEN group. The EPEN group showed significantly fewer incidences of postoperative pancreatic fistulas, as well as a significantly shorter length of hospitalization than the LPEN group. There were no significant differences in the incidences of other postoperative complications between the two groups, such as delayed gastric emptying, surgical site infection, cholangitis, and small bowel obstruction.

CONCLUSION: EPEN is a safe and beneficial

INTRODUCTION

Postoperative nutritional support was shown to reduce the incidence of complications and/or to shorten the hospitalization period^[1,2]. Recently, early postoperative enteral nutrition (EPEN) has been proposed as the novel method for nutritional support after surgery, especially after gastric and colorectal resection^[3-9]. Several studies suggested that EPEN possibly improves also the postoperative outcome of patients after duodenohepaticoduodenectomy (DHP)^[10-13], which is one of the most invasive operations in the upper abdominal surgery with a high incidence of postoperative complications^[14-19]. Whereas, EPEN has been introduced with complications such as troubles of jejunal feeding tube and delayed gastric emptying^[10-13,20,21]. Taken together, the overall benefit of EPEN after DHP remains controversial.

The purpose of the present study was to evaluate the influence of EPEN on the incidence of postoperative complications. Moreover, by analyzing a variety of clinical parameters including laboratory data, body mass index (BMI), and the duration of hospitalization, we attempted to determine which method of postoperative nutritional support, enteral or non-enteral, was more advantageous in the total management of patients who had undergone DHP.

MATERIALS AND METHODS

Materials

We investigated a total of 39 patients who had undergone

Table 1 Patient profiles

| | LPEN group (n = 23) | EPEN group (n = 16) | P value |
|------------------------|------------------------|------------------------|---------|
| Demographics | | | |
| Age (range) | 67.2 yrs (42-82) | 68.0 yrs (54-81) | 0.8137 |
| Gender: male | 65.2% | 56.3% | 0.8168 |
| Underlying disease (%) | | | 0.6206 |
| Cholangiocarcinoma | 9 (39.1) | 2 (12.5) | |
| Pancreatic carcinoma | 6 (26.1) | 8 (50.0) | |
| IPMN | 4 (17.4) | 3 (18.8) | |
| CPV | 3 (13.1) | 1 (6.2) | |
| Others | 1 (4.3) | 2 (12.5) | |

IPMN: Intraductal papillary mucinous neoplasm; CPV: Carcinoma of the papilla of Vater; Other diseases: Gastrointestinal stromal tumor, primary sclerosing cholangitis, and pancreatic metastasis of renal cell carcinoma

DHP for a peri-ampullary mass from 2000 to 2005 at Kochi Medical School, including 24 men and 15 women (mean age of 67.5 years; 43-82 years). Among these 39 patients, there were 14 cases of pancreatic invasive ductal carcinoma, 11 of cholangiocarcinoma, 7 of intraductal papillary mucinous neoplasms, 4 of carcinoma of the papilla of Vater, 1 of gastrointestinal stromal tumor, 1 of primary sclerosing cholangitis, and 1 of metastatic renal cell carcinoma (Table 1). All patients underwent a complete perioperative physical examination and laboratory investigations. Moreover, a variety of relevant parameters regarding the operative procedure and anesthesia were recorded in all cases.

Operative procedure

In all patients, the reconstructive technique was used to anastomose the pancreas first, followed by the hepatic duct and the duodenum with a Braun anastomosis. The pancreatic-enteric anastomosis was performed as a pancreaticojejunostomy in an end-to-side fashion. In patients of the EPEN group, a feeding jejunostomy catheter was placed at the end of surgery and before closing the wound through the anterior wall of the stomach using a modified Witzel technique. Furthermore, in all cases of pylorus-preserving pancreaticoduodenectomy (PpPD), the gastrostomy tube was inserted from the afferent loop of the jejunum into the stomach for the purpose to prevent delayed gastric emptying.

Postoperative nutrition

We determined the amount of calories required for postoperative nutrition according to the Harris-Benedict equation^[22]. Some patients received total parenteral nutrition and then started to have oral intake of nutrition usually between POD 7 and 14, as determined late postoperative enteral nutrition (LPEN) group. The second group of patients started to have EPEN through a catheter-feeding jejunostomy on POD1 (within 24 h after surgery), as determined EPEN group. Enteral feeding was started at a rate of 20 mL/h and gradually increased by 10 mL/h a day up to the final rate (70 mL/h).

Laboratory and clinical investigations

The operation time, blood loss volume, and amount

Table 2 Operative characteristics

| GroupCharacteristics | LPEN group (n = 23) | EPEN (n = 16) | P value |
|-------------------------|------------------------|------------------|---------|
| Operative procedure (%) | | | 0.0371 |
| PpPD | 12 (52.2) | 14 (87.5) | |
| PD | 11 (47.8) | 2 (12.5) | |
| Demographics (range) | | | |
| Operative time (min) | 516 (360-765) | 509 (370-605) | 0.5417 |
| Blood loss volume (mL) | 1 014 (400-1 650) | 908 (400-1 600) | 0.0593 |
| Transfused patients (%) | 17 (73.9) | 8 (53.3) | 0.3384 |
| RC-MAP (U) | 1.9 (0-10) | 1.1 (0-6) | 0.0599 |
| FFP (U) | 9.2 (0-40) | 5.3 (0-10) | 0.1220 |
| IOR (%) | 2 (8.7) | 7 (43.8) | 0.0190 |

PD: Pancreaticoduodenectomy; PpPD: Pylorus-preserving pancreaticoduodenectomy; RC-MAP: Red cells in mannitol-adenine-phosphate solution; FFP: Fresh frozen plasma; IOR: intra-operative radiotherapy.

of blood transfusion during and after the surgery were carefully recorded. Samples for laboratory investigations were taken on POD 1, 4, 6, and 14. The laboratory parameters included serum levels of total protein, albumin, total bilirubin, cholinesterase, alanine transaminase, aspartate transaminase, lactate dehydrogenase, alkaline phosphatase, gamma-glutamyl transpeptidase, amylase, urea nitrogen, and creatinine. The BMI was measured before surgery and on POD 6 and 14. Postoperative complications, including surgical site infection, leakage from anastomosis, pancreatic fistula, cholangitis, small bowel obstruction and delayed gastric emptying, were carefully monitored every day. The duration of hospitalization was defined as the time from the day of the surgery to the day of discharge. The progress of all patients, following their discharge from hospital, was monitored by our hospital.

Pancreatic fistula

We determined the occurrence of pancreatic fistula based on the following criteria: the concentrations of amylase and lipase in the drainage fluid being three times higher than that in the serum on consecutive PODs, and the drainage volume being more than 10 mL/d. Amylase and/or lipase concentrations in the serum and drainage fluid were checked on POD 1, 3, 4, 5, and 7, and twice a week thereafter^[23-25].

Statistical analysis

We tested for statistical significance using the χ^2 test, the Fisher's exact test and the *t* test. $P < 0.05$ was considered statistically significant. Where appropriate, values were expressed as mean \pm SD.

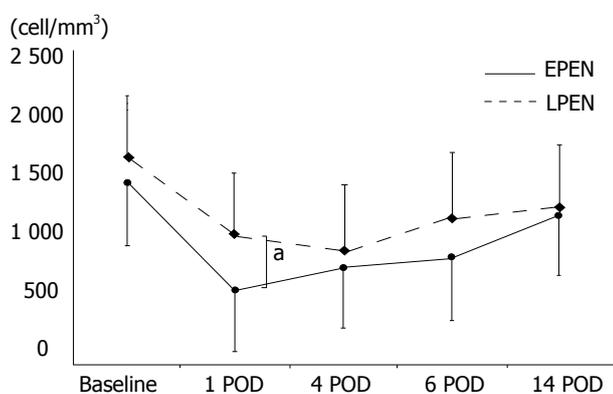
RESULTS

We retrospectively reviewed 39 patients who had undergone DHP between 2000 and 2004 at Kochi Medical School, and subdivided them into two groups, 23 patients in the LPEN group and 16 patients in the EPEN group (Table 1). Hospital mortality was 2.6%. There were no significant differences between the two groups in age,

Table 3 Postoperative outcome of patients with pancreatic surgery

| Characteristics | LPEN group (n = 23) | EPEN group (n = 16) | P value |
|----------------------------------|------------------------|------------------------|---------|
| BMI | | | |
| Baseline | 21.75 ± 3.16 | 22.58 ± 2.60 | 0.8044 |
| POD 6 | 20.43 ± 2.67 | 2.40 ± 2.59 | 0.0305 |
| POD 14 | 20.14 ± 2.50 | 22.38 ± 2.55 | 0.0111 |
| Postoperative complications (%) | | | |
| Anastomotic leakage | 0 (0.0) | 0 (0.0) | NS |
| Surgical site infection | 2 (8.7) | 2 (12.5) | 0.8797 |
| Pancreatic fistula | 9 (39.1) | 1 (6.3) | 0.0279 |
| Ventral hernia | 0 (0.0) | 1 (6.3) | 0.4103 |
| Cholangitis | 6 (26.1) | 5 (31.3) | 0.9926 |
| Small bowel obstruction | 1 (4.3) | 1 (6.3) | 0.6362 |
| Delayed gastric emptying | 1 (4.3) | 2 (12.5) | 0.5571 |
| Length of hospitalization (days) | 44.3 ± 19.0 | 31.7 ± 8.8 | 0.0011 |

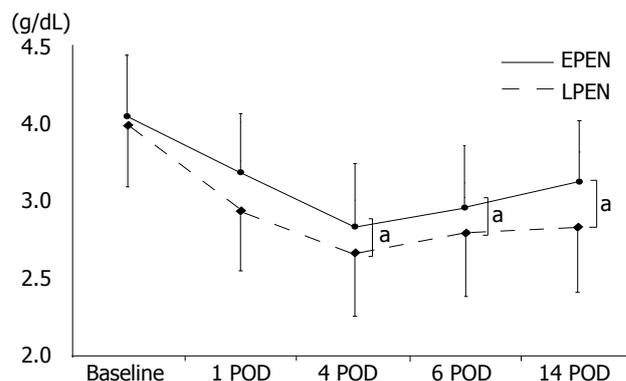
NS: not significant

**Figure 1** Peripheral lymphocyte coun. ^a*P*<0.05 vs LPEN.

gender, and the incidence of underlying diseases.

PpPD was carried out in 26 cases and PD in 13 cases. The frequencies of PpPD and intra-operative radiotherapy (IOR) were significantly higher in the EPEN group than in the LPEN group, because we started IOR for all patients with pancreatic carcinoma in 2002 (*P* < 0.05). There were no significant differences between the two groups in other parameters (Table 2) including operation time, blood loss volume, and the proportion of patients who received blood transfusion including red blood cells in mannitol-adenine-phosphate solution (RC-MAP) and fresh frozen plasma (FFP).

The baseline preoperative values of all laboratory parameters were comparable between the two groups. In the postoperative course, however, several parameters showed a significant difference. The lymphocyte count decreased immediately after the operation (at POD 1) in both groups (Figure 1), which was more obviously seen in the EPEN group compared to the LPEN group (*P* < 0.05), probably due to the higher incidence of IOR (Table 2). However, during POD 1 and 4, lymphocyte count in the LPEN group continuously decreased, but increased in the EPEN group. There was no significant difference between the two groups in lymphocyte count at POD 4 and

**Figure 2** Serum albumin level. ^a*P*<0.05 vs LPEN.

thereafter. The serum levels of albumin (Figure 2) and the total protein (data not shown) decreased immediately after the operation in both groups. However, albumin levels at POD 4, 6, and 14 (Figure 2) and total protein levels at POD 6 and 14 (data not shown) in the EPEN group were significantly higher than those in the LPEN group. These findings in the restoration of nutritional parameters were consistent with the alteration in BMI. Loss of BMI was significantly suppressed in the EPEN group as compared to the LPEN group (Table 3).

None of the cases had a complication of anastomotic leakage. The EPEN group had a significantly lower incidence of pancreatic fistula than the LPEN group (*P* < 0.05). There was no significant difference between the two groups in the incidence of other postoperative complications, including surgical site infection, ventral hernia, cholangitis, small bowel obstruction, and delayed gastric emptying (Table 3). Finally, the EPEN group also required a significantly shorter length of hospital stay than the LPEN group (*P* < 0.01).

DISCUSSION

In the present study, we employed 39 patients who underwent DHP and subdivided them into two groups according to the procedures of the postoperative nutritional support: the EPEN and LPEN groups. There was no significant difference in baseline profiles between the two groups except for the following two parameters, which should be considered in the analysis of the results. First, the number of patients with PD was greater in the LPEN group than in the EPEN group. EPEN after DHP was introduced in our department in 2002, in order to assess its utility. Until then, almost all patients who underwent PD for a peri-ampullary mass were postoperatively administered with total parenteral nutrition. However, we believed that there were no significant differences in the surgical procedure because two surgeons performed the pancreatic surgery in this study period. Second, the number of patients with IOR was much greater in the EPEN group than the LPEN group. The difference was caused by the fact that we started IOR for all patients with pancreatic carcinoma in 2002^[26-28].

Although the serum levels of albumin and total protein

dropped remarkably in all the patients after the operation, they recovered quickly in the EPEN group, and were significantly higher than those in the LPEN group at the early postoperative stage. Consistent with these findings, loss of BMI was significantly suppressed in the EPEN group as compared to the LPEN group. These findings indicate that EPEN modulates a metabolic response, favoring the synthesis of proteins. We believe that nutritional improvement observed in the EPEN group was not influenced by a large proportion of patients with PpPD (87.5%), since PpPD has been reported to provide a long-term nutritional support for operated patients but has not any benefit for nutritional status in early postoperative stage^[29].

Furthermore, the lymphocyte count fell immediately after surgery in the EPEN group probably due to the IOR, resulting in the significantly lower level as compared to the LPEN group ($P < 0.05$). However, the lymphocyte count in the EPEN group increased thereafter, and reached at the similar level observed in the LPEN group at POD 14. These findings suggest that the administration of EPEN not only improves the nutritional status but also improves whole-body protein kinetics.

There was no significant difference between two groups in the incidence of infectious complications, such as surgical site infection and cholangitis, and also noninfectious complications, such as ventral hernia and small bowel obstruction. Although the EPEN group contained a significantly greater number of patients with PpPD than the LPEN group, there were no significant differences in the occurrence of delayed gastric emptying between the two groups. The incidence of delayed gastric emptying has been reported in 7-36% of patients with DHP^[18,30-34]. Usually, the delayed gastric emptying is more frequently seen in patients with PpPD than in patients with PD, and is typically associated with prolonged hospitalization. In our study, tube gastrostomy was created in all patients who underwent PpPD, and thus patients with delayed gastric emptying had no vomiting.

Pancreatic fistula is considered as a major postoperative complication of DHP and has been reported in 5-24% of patients with DHP^[23,35-38]. In our study, surprisingly, the incidence of pancreatic fistula was significantly lower in the EPEN group (6.3%) than in the LPEN group (39.1%). There have been some concerns that EPEN could increase the possibility of pancreatic fistulae because of its stimulatory effect on exocrine pancreatic secretion. Our data show that EPEN has no bad influence upon the occurrence of pancreatic fistula but rather works to prevent it.

In conclusion, EPEN is a safe and beneficial procedure for patients who have undergone DHP. EPEN improves early postoperative outcomes, including nutritional status and whole-body protein kinetics. Furthermore, EPEN contributes to a significantly lower incidence of pancreatic fistula, resulting in a shorter duration of hospitalization compared to the LPEN group. Based on these findings, EPEN can provide regular postoperative nutritional support following DHP.

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RAPID COMMUNICATION

L1 is a potential marker for poorly-differentiated pancreatic neuroendocrine carcinoma

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Abstract

AIM: To determine the expression of L1 in pancreatic neuroendocrine tumor and to correlate it with WHO classification of this tumor.

METHODS: We retrospectively analyzed L1 expression in 63 cases of pancreatic neuroendocrine tumor by immunohistochemistry on paraffin sections of primary tumors or metastases. Staining was performed by peroxidase technique with monoclonal antibody UJ127.11 against human L1. All tumors were classified according to WHO classification as well-differentiated neuroendocrine tumors and carcinomas or poorly-differentiated neuroendocrine carcinomas.

RESULTS: L1 was detected in 5 (7.9%) of 63 pancreatic neuroendocrine tumors. Four (44.4%) of 9 poorly-differentiated carcinomas expressed L1. In contrast, only 1 (1.9%) of 54 well-differentiated tumors or carcinomas was positive for L1. No expression was found in Langerhans islet cells of normal pancreatic tissue. Cross table analysis showed a significant association between L1 expression and classification of neuroendocrine tumors of the pancreas ($P < 0.01$).

CONCLUSION: L1 is specifically expressed in poorly-differentiated pancreatic neuroendocrine carcinomas that are known to have the worst prognosis. L1 might be a marker for risk prediction of patients diagnosed with

INTRODUCTION

Neuroendocrine tumors of the gastroenteropancreatic axis are rare and characterized by significant phenotypic differences. They can present themselves as benign or highly malignant and their clinical behavior is very heterogeneous. They are considered to originate from cells of the disseminated neuroendocrine cell system^[1]. Most endocrine tumors of the pancreas are well-differentiated neuroendocrine tumors or carcinomas. Frequently, they appear to be malignant with the exception of insulinoma^[2]. Fifty percent to sixty percent of these tumors are functionally active and secrete insulin, gastrin, vasoactive intestinal polypeptide (VIP), glucagon or other rare hormones and consequently cause characteristic syndromes. The most important criteria of malignancy include a tumor size of more than 2 cm, angioinvasion and proliferative activity of more than 2% of the tumor cells apart from metastases to the regional lymph nodes and the liver or invasion of adjacent organs^[3,4]. Neuroendocrine tumors of the pancreas are classified according to the WHO classification into well-differentiated tumors and carcinomas or poorly-differentiated carcinomas^[4,5]. Poorly-differentiated neuroendocrine carcinomas of the pancreas are highly malignant with a bad prognosis^[3].

Neoplastic cells frequently re-express adhesion molecules involved in cell migration during tissue morphogenesis and fetal development^[6]. The L1 cell adhesion molecule (CD171) is a 200-220 ku type I glycoprotein of the immunoglobulin superfamily and plays a role in development of the nervous system by regulating cell interactions, includ-

ing neuronal migration^[7,8]. L1 also mediates neuron-neuron adhesion, neurite outgrowth on Schwann cells, neurite fasciculation and myelination^[7]. L1 undergoes homophilic L1-L1 binding and heterophilic interactions with several ligands such as integrins^[9,10]. L1 is expressed also in hematopoietic and certain epithelial cells as well as in a variety of tumors, such as of neuroblastomas, melanomas, small cell lung cancer and breast carcinomas^[11-15]. Metalloproteinase (ADAM10) also triggers cell migration and cleaves L1 from the tumor cell surface^[8,12,16-18]. Recently, it was reported that expression of L1 has a prognostic significance in ovarian and uterine carcinomas and is associated with metastasis of melanomas^[19,20]. Furthermore, L1 is expressed in neuroendocrine tumors of the skin^[21]. Up-regulation of L1 expression has also been observed in malignant pleural mesotheliomas and malignant peripheral nerve sheath tumors by microarray expression profiling^[22,23].

The aim of this study was to determine the expression of L1 in neuroendocrine tumors of the pancreas and its relation to tumor stage of this heterogeneous cancer type. We detected the expression of L1 in 4 (44.4%) of 9 poorly differentiated pancreatic neuroendocrine carcinomas. However, only 1 (1.9%) of 54 well-differentiated tumors or carcinomas was positive for L1. Cross table analysis showed a significant correlation between L1 expression and poorly-differentiated neuroendocrine carcinomas. Our data indicate that L1 is a specific marker for malignant phenotype of pancreatic neuroendocrine carcinomas.

MATERIALS AND METHODS

Study design and patients

The study was approved by the Ethics Committee of the Chamber of Physicians in Hamburg, Germany. Written informed consent was obtained from all patients for use of the resected samples. For this study, 63 patients with pancreatic neuroendocrine tumors were chosen retrospectively. We selected patients on the basis of availability of tissues and did not stratify them due to rare occurrence and different treatment strategies. Forty-seven primary tumors of the pancreas and 38 metastases (21 from liver, 16 from lymph nodes and 1 from spleen) were available. All tumors were categorized into 3 groups according to WHO classification of 2 000 into well-differentiated tumors (grade 1a) and carcinomas (grade 1b) or poorly-differentiated neuroendocrine carcinomas (grade 2)^[4]. Briefly, this classification was based on tumor size, angioinvasion, proliferating activity, histological differentiation and hormonal activity. All data including sex, histology, depth of tumor invasion, lymph node metastasis, tumor type and disease stage were obtained from the clinical and pathological records.

Immunohistochemical staining and evaluation of expression

Immunohistochemical staining was performed for 5- μ m thick sections of formalin-fixed and paraffin-embedded tissues placed on pre-coated slides with 3-triethoxysilylpropylamin (Merck, Darmstadt, Germany). After deparaffinization with Rotihistole (Merck) and rehydration in ethanol and TBS (0.05mol/L, pH 7.6) containing 10 g/L Tween 20 (Sigma, Deisenhofen, Germany), tissue sections

were pre-treated for 30 min in 10 g/L ammonium chloride (NH₄Cl) in TBS, for 15 min in 0.05 mol/L glycine/TBS and then boiled with ChemMate[®] target retrieval solution (Dako, Hamburg, Germany) in a microwave oven according to the manufacturer's instructions. Staining was performed with the peroxidase method (HRP-AEC System, Cell and Tissue Staining Kit; R&D Systems, Minneapolis, MN, USA). The primary antibody, a murine anti-human L1 monoclonal antibody (IgG₁, clone UJ127) (NeoMarkers, Fremont, CA, USA) binding to the extracellular domain of this molecule, was diluted at 1:50 in antibody diluent (Dako) and slides were incubated overnight in a humidity chamber at 4°C^[24]. For each sample one slide, a control section was incubated with irrelevant murine monoclonal IgG₁ (MOPC21; Sigma) as a negative control to determine the unspecific binding. All washing steps were done with TBS containing 10 g/L Tween 20. Counterstaining was performed with Haemalaun Mayer (Merck) for 30 s followed by Mayer's haematoxylin solution (Merck) for 7 min. At last, slides were covered with coverslips with aqueous mounting medium (Aquatex[®]; Merck). Specimens were considered immunopositive for L1 when >20% of the tumor cells had clear evidence of immunostaining. Peripheral nerves present in almost all sections served as internal positive controls. Langerhans islet cells were negative for L1 in normal pancreatic tissue. Immunohistochemical analysis and scoring of the sections were performed by two independent investigators and one pathologist in a blinded fashion. Two sections were scored differently and in these cases the opinion of the pathologist was decisive.

Statistical analysis

We used SPSS for Windows (SPSS Inc., Chicago, IL USA) for statistical analysis. The immunostaining results of L1 and WHO classification of neuroendocrine tumors of the pancreas were calculated using a cross table and statistical analysis was performed with *F*-test. *P*<0.05 was considered statistically significant.

RESULTS

Characteristics of the patients

Sixty-three patients suffering from pancreatic neuroendocrine tumor were included in the study. Characteristics of the patients are listed in Table 1. Briefly, the median age of the study population was 57 years, 32 (50.8%) patients were male and 31 (49.2%) female. According to WHO classification for neuroendocrine tumors of the gastroenteropancreatic axis, 50 (79.4%) were classified as well-differentiated neuroendocrine tumors (grade 1a), 4 (6.3%) as well-differentiated carcinomas (grade 1b) and 9 (14.3%) as poorly-differentiated neuroendocrine carcinomas (grade 2), being the most malignant phenotype. Eleven (17.5%) tumors showed hormone production and 6 (9.5%) of 63 patients suffered from endocrine neoplasia (MEN) type I.

Immunohistochemical analysis of L1 in pancreatic neuroendocrine tumors

L1 expression was determined by immunohistochemical analysis in samples from 63 pancreatic neuroendocrine tu-

Table 1 Characteristics of the patients and levels of L1 expression *n*(%)

| Variable | Patients | L1-positive tumors |
|---|-----------|--------------------|
| Total | 63 | 5 (7.9) |
| Male | 32 (50.8) | 4 (12.5) |
| Female | 31 (49.2) | 1 (3.2) |
| <i>WHO classification of neuroendocrine pancreatic tumour</i> | | |
| Well-differentiated neuroendocrine tumour (grade 1a) | 50 (79.4) | 1 (2.0) |
| Well-differentiated neuroendocrine carcinoma (grade 1b) | 4 (6.3) | 0 |
| Poorly-differentiated neuroendocrine carcinoma (grade 2) | 9 (14.3) | 4 (44.4) |
| <i>Hormone production</i> | | |
| Yes | 11 (17.5) | 0 |
| No | 52 (82.5) | 5 (9.6) |
| <i>Multiple endocrine neoplasia (MEN)-I</i> | | |
| MEN-I | 6 (9.5) | 0 |
| No MEN-I | 57 (90.5) | 5 (8.8) |

Table 2 Correlation of L1 expression with WHO classification

| WHO classification | L1-negative | L1-positive | Total |
|---|-------------|-------------|-------|
| Well-differentiated neuroendocrine tumors and | 53 | 1 | 54 |
| Well-differentiated neuroendocrine carcinomas (grade 1a and 1b) | | | |
| Poorly-differentiated neuroendocrine carcinomas (grade 2) | 5 | 4 | 9 |
| Total | 58 | 5 | 63 |

$P < 0.01$ by Fisher's test (two-sided)

mor patients. Forty-seven primary tumors of the pancreas and 38 metastases (21 from liver, 16 from lymph nodes and 1 from spleen) were available and immunostained. In 18 patients, both primary tumor and metastases (12 from lymph nodes, 3 from liver, 2 from both lymph nodes and liver, 1 from liver and spleen) were investigated. In 16 patients, only metastases were available (14 from liver, 1 from lymph nodes, 1 from both lymph nodes and liver). No differences in terms of positivity or negativity of L1 expression were detected between primary tumor and metastases in any patient. Figure 1 shows the representative negative and positive staining patterns for L1 of pancreatic neuroendocrine tumors. Staining was not detected in normal pancreatic islet cells.

Five (7.9%) of 63 cases were L1-positive (Table 1). were stained. The remaining 58 (92.1%) patients were negative for L1. According to the WHO classification, 4 (44.4%) of 9 poorly-differentiated neuroendocrine carcinomas (grade 2) were L1-positive. Only 1 (1.9%) of 54 well-differentiated neuroendocrine tumor samples (grade 1a) was L1-positive. None of the 4 well-differentiated neuroendocrine carcinomas (grade 1b) was positive for L1. Although only 9 poorly-differentiated pancreatic neuroendocrine carcinomas (grade 2) were available, these results showed that L1 was specifically expressed in poorly dif-

ferentiated tumors. Forty-four point four percent of these most highly malignant tumors were positive for L1 compared to 1.9% in the group of well-differentiated neuroendocrine tumors or carcinomas (grade 1a and 1b).

Correlation between L1 expression and WHO classification of tumor

A significant correlation between L1 expression and well-differentiated neuroendocrine tumor (grade 1a) and (grade 1b) or poorly-differentiated neuroendocrine carcinoma (grade 2) was found by Fisher's exact test ($P < 0.01$, Table 2).

DISCUSSION

Pancreatic neuroendocrine tumors or carcinomas are rare and clinically very heterogeneous. Neuroendocrine-specific molecules are positive markers of endocrine differentiation in tumor cells^[1]. Little is known about the molecular differences between benign and malignant phenotypes of neuroendocrine tumors of the pancreas. Cell adhesion molecules, such as L1, have been repeatedly implicated in tumor progression and metastasis. In this study, we determined L1 expression in 7.9% of 63 cases of pancreatic neuroendocrine tumor. Since L1 is not expressed in normal Langerhans islet cells, which are believed to be the precursor cells of pancreatic neuroendocrine tumors, an up-regulation of L1 expression in tumor cells may be associated with tumorigenesis in this tumor type.

We used immunohistochemical analysis for detection of L1 in tumor cells. Because the optimal cutpoint approach has some limitations in statistical evaluation of prognostic factors, we chose a cutpoint of 20% L1-positive tumor cells in the analysed cell population, achieving an easy discrimination of immunostained tumor tissue as proposed by Altman and colleagues^[25].

In our study, there were 9 patients with poorly-differentiated neuroendocrine carcinoma according to WHO classification (grade 2). Out of these, 4(44.4%) had positive L1 expression. L1 In contrast, only 1 (1.9%) of 54 well-differentiated tumors and carcinomas (grade 1a and 1b) was positive for L1. Statistical analysis showed a significant correlation between L1 expression and poor differentiation of pancreatic neuroendocrine tumors (grade 2), suggesting that L1 is a marker for poorly differentiated and highly malignant pancreatic neuroendocrine carcinomas. Although the number of L1-positive tumors in our study was too low for a final conclusion, the number of L1-negative tumors supports our notion that L1 is a marker for malignancy of this tumor entity.

These observations are in agreement with previous studies correlating expression of L1 in different tumors of neuroectodermal origin, such as melanomas or uterine and ovarian carcinomas, with malignancy and poor prognosis^[15,19-21,26]. Our results prove that expression of L1 can also be found in another neuroendocrine tumor, namely the neuroendocrine tumor of the pancreas.

Further studies are needed to determine the potential prognostic value of L1 expression in patients suffering from pancreatic neuroendocrine carcinomas. Our data also indicate that downregulation of L1 expression by antisense technologies may be used as a therapeutic method.

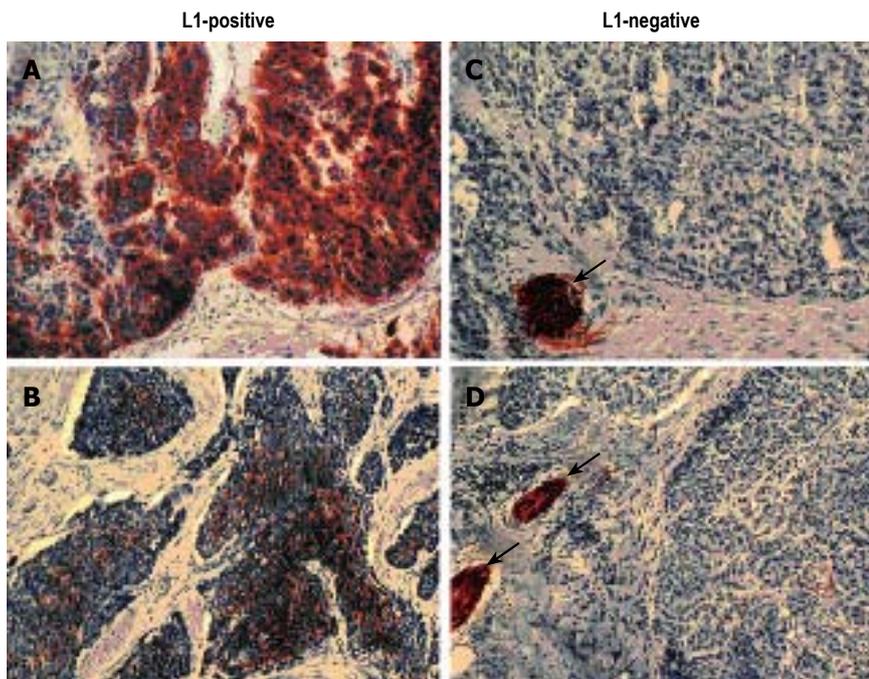


Figure 1 L1 expression in pancreatic neuroendocrine tumours or carcinomas. Immunohistochemical staining was performed by peroxidase method using monoclonal antibody UJ.127 against L1. Poorly-differentiated L1-positive pancreatic neuroendocrine carcinomas (grade 2; A and B) were shown in comparison to well-differentiated L1-negative tumours (grade 1a; C and D). Peripheral nerves (arrows) stained in (C, D) served as internal positive controls (Magnification $\times 200$ (A and C) and $\times 400$ (B and C)).

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Risk factors for immediate post-operative fatal recurrence after curative resection of hepatocellular carcinoma

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tumor size > 6.5 cm, and microvascular invasion. The high risk patients with two or more risk factors should be the candidates for various adjuvant clinical trials.

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Abstract

AIM: To investigate the clinicopathological risk factors for immediate post-operative fatal recurrence of hepatocellular carcinoma (HCC), which may have practical implication and contribute to establishing high risk patients for pre- or post-operative preventive measures against HCC recurrence.

METHODS: From June 1994 to May 2004, 269 patients who received curative resection for HCC were reviewed. Of these patients, those who demonstrated diffuse intra-hepatic or multiple systemic recurrent lesions within 6 mo after surgery were investigated (fatal recurrence group). The remaining patients were designated as the control group, and the two groups were compared for clinicopathological risk factors.

RESULTS: Among the 269 patients reviewed, 30 patients were enrolled in the fatal recurrence group. Among the latter, 20 patients showed diffuse intra-hepatic recurrence type and 10 showed multiple systemic recurrence type. Multivariate analysis between the fatal recurrence group and control group showed that pre-operative serum alpha-fetoprotein (AFP) level was greater than 1 000 µg/L ($P=0.02$; odds ratio=2.98), tumor size greater than 6.5 cm ($P=0.03$; OR=2.98), and presence of microvascular invasion ($P=0.01$; OR=4.89) were the risk factors in the fatal recurrence group. The 48.1% of the patients who had all the three risk factors and the 22% of those who had two risk factors experienced fatal recurrence within 6 mo after surgery.

CONCLUSION: Three distinct risk factors for immediate post-operative fatal recurrence of HCC after curative resection are pre-operative serum AFP level > 1 000 µg/L,

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the common causes of cancer death among Koreans, and it is also one of the frequently occurring cancers worldwide. Surgical resection of the liver has been one of the mainstays in the curative treatment of this cancer. Recent advances in anatomical knowledge of liver, surgical skills and instruments, intra- and post-operative management techniques have led to a marked reduction in post-operative mortality rates. However, recurrence after curative partial hepatectomy for HCC occurs in approximately 70% of patients^[1-3], and even after careful selection of relatively early disease patients for liver transplantation (OLT) according to the Milan selection criteria. It has been reported that the recurrence rate is about 20%^[4]. This finding is considered to be the most significant risk factor for the survival of the patient.

The pattern of recurrence after curative surgery for HCC is variable. Among these diverse patterns of recurrence, diffuse intra-hepatic recurrence and multiple systemic recurrences are thought to be fatal not only because there is no effective treatment strategy, but also these recurrence patterns inevitably mean a short remaining survival time. Moreover, the development of such fatal recurrences soon after surgical resection may indicate the presence of multiple intra-hepatic micro-metastases or systemic dissemination and colonization of HCC cells at the time of surgery. But present clinical technology does not allow pre-operative determination of micro-metastatic lesions, and therefore the available

approach at present is the determination of risk factors for immediate post-operative fatal recurrence.

Numerous studies have investigated the recurrence of HCC after partial hepatectomy and have reported a number of risk factors for recurrence. But the application of such risk factors in the practical field for surgeons is difficult, which may be due to the fact that the limitation of application of these risk factors is confusing, and sometimes too broad in perspective. In this study, we attempted to elucidate the risk factors involved in the recurrence of HCC soon after partial hepatectomy, which entail more practical implications for the liver surgeon.

MATERIALS AND METHODS

Patients

From June 1994 to May 2004 for a period of 10 years, the medical records of 322 HCC patients who received partial hepatectomy at the Department of Surgery, Ajou University Hospital were reviewed. These patients were divided into the fatal recurrence group and the control group. The inclusion criteria of the fatal recurrence group were the patients who had diffuse intra-hepatic recurrence or multiple systemic recurrence within 6 mo after curative surgical resection of HCC. The rest were designated as the control group, and risk factors for the fatal recurrence group were analyzed.

Among the total 322 patients, the following were excluded from this study: patients who died within 6 mo after surgery due to reasons other than recurrence; patients who were lost to follow-up within 6 mo after surgery; patients whose histopathologic examination showed fibrolamellar or combined cholangiohepatocellular carcinoma pathologies; and patients who received non-curative resection.

Methods

The criteria for curative liver resection followed by the authors were the General Rules for the Study of Primary Liver Cancer Guidelines set up by the Korean Liver Cancer Study Group^[5]. In these guidelines, the definition of curative surgery for HCC is classified as A1, A2, and B: "A1 = tumor size < 2 cm and no residual tumor after resection, without vascular or ductal invasion; A2 - tumor size 2 - 5 cm and no residual tumor after resection, without vascular or ductal invasion; and B = no residual tumor after resection, but not included in A1 or A2". Those who were regarded as non-curative resection were the patients with gross evidence of residual tumor, tumor invasion into the 1st order branch of the portal vein or main portal vein, tumor invasion of the left, right, middle, and inferior right or short hepatic vein and tumor invasion into 2nd or 1st order branches of the intra-hepatic bile duct or common hepatic duct.

Pre-operative radiological evaluation included abdominal ultrasonography, abdominal computerized tomography (CT) scans and hepatic angiography. Magnetic resonance imaging (MRI) and/or positron emission tomography (PET) scan were also conducted when deemed necessary by the physician in charge. To evaluate the residual liver function, all patients received the dye

retention test using indocyanine green (ICG) in addition to the general chemistry tests. Also, to accurately assess the degree of curative surgery, all patients underwent intra-operative ultrasonography evaluation.

Post-operative follow-up consisted of monthly serum alpha-fetoprotein (AFP) level examination, and abdominal ultrasonography and plain chest films at every 3 mo. Abdominal CT was performed every 6 mo. The follow-up continued until the patient died of disease. If the serum AFP level did not decrease to normal level or abnormally increased or if one or more suspicious recurrent lesions were detected on ultrasonography during the post-operative follow-up, the results were then further confirmed by abdominal CT and/or chest CT and/or PET scan and hepatic angiography.

Diffuse intra-hepatic recurrence was defined as five or more recurrent lesions in the remnant liver as demonstrated by hepatic angiography^[6], and multiple systemic recurrence was defined as two or more systemic recurrent lesions without evidence of intra-hepatic recurrence, as shown by imaging studies.

Twenty-four variables were compared between the fatal recurrence group and control group. The patient factors were: age, sex, presence of symptoms, hepatitis B antigen status, hepatitis C antibody status, Child's classification, pre-operative serum AST and ALT levels, pre-operative ICG R-15 and serum AFP levels, the grade of hepatitis, and stage of cirrhosis in non-tumor liver tissues. The tumor factors that were investigated were: size of tumor, single or multiple lesions, tumor growth patterns, presence of tumor capsule and invasion of tumor capsule, gross evidence of vascular or bile duct tumor involvement, formation of intra-tumor septum, tumor invasion of the Glisson's capsule, presence of microvascular invasion and the Edmond-Steiner grade of tumor cells. Successful anatomic resection and tumor margins of more than 1 cm were defined as surgical factors. The anatomical resection was defined as complete segmental resection of the Couinaud's segment system. The pathological review was performed by an experienced pathologist (Kim) in the Department of Pathology in our institute.

Statistical analysis

Univariate statistical analysis was performed using χ^2 test and Student's *t*-test, and multivariate analysis was carried out using logistic regression analysis.

RESULTS

Among the 322 patients, 53 patients were excluded from this study: 7 patients who died within 6 mo after surgery due to reasons other than recurrence (3 of 7 patients were post-operative in-hospital mortality); 3 patients who were lost to follow-up within 6 mo after surgery; 10 patients whose histopathologic examination showed fibrolamellar or combined cholangiohepatocellular carcinoma pathologies; and 33 patients who were regarded as non-curative resection.

Among the 269 patients who were included in this study, the mean follow-up period was 32.2 ± 25.6 mo (range, 4 - 107 mo). Of the 269 patients, 42 (15.6%) experienced

Table 1 Univariate analysis of host factors for immediate post-operative diffuse intra-hepatic recurrence or multiple distant recurrence after curative resection for HCC

| Host factor | Patients (%) or (mean \pm SD) | | P |
|---|---------------------------------|-------------------|------|
| | Fatal recurrence group | Control group | |
| Sex, male (%) | 76 | 74 | NS |
| Mean age(yr) | 49.2 \pm 9.9 | 52.1 \pm 10.4 | NS |
| HCC-related symptom | 50 | 31 | 0.04 |
| Child's classification A/B/C | 90/10/0 | 90/9/1 | NS |
| ICG R-15 (%) | 14.6 \pm 11.3 | 13.4 \pm 8.6 | NS |
| Serum ALT (nkat/L) | 1185 \pm 1285 | 1034 \pm 944 | NS |
| Serum AST (nkat/L) | 1649 \pm 1259 | 1125 \pm 1539 | NS |
| HBs Ag positivity | 77 | 74 | NS |
| HCV Ab positivity | 13 | 8.4 | NS |
| Serum AFP level (μ g/L) | 10 447.4 \pm 12 931.9 | 23045 \pm 6 660 | 0.00 |
| Histologic grade of hepatitis 0, 1, 2/3, 4 ¹ | 86.7/13.3 | 92.8/7.2 | NS |
| Histologic stage of cirrhosis 0, 1, 2/3, 4 ¹ | 15.8/84.2 | 19.2/80.8 | NS |

¹Data in each parameter showed less than 2% missing rate, but histologic grade of hepatitis and histologic stage of cirrhosis showed 9% and 10.5% data missing rate, respectively.

Table 2 Univariate analysis of tumor and surgical factors for immediate post-operative diffuse intra-hepatic recurrence or multiple distant recurrence after curative resection of HCC

| | Patients(%) or (mean \pm SD) | | P |
|--|--------------------------------|---------------|------|
| | Fatal recurrence group | Control group | |
| Tumor factors | | | |
| Tumor size (cm) | 10.1 \pm 6.2 | 4.8 \pm 3.2 | 0.00 |
| Tumor growth pattern, Eg ¹ /Ig ² | 36.7/63.3 | 7.9/92.1 | 0.00 |
| Multiple tumors | 60.0 | 28.5 | 0.01 |
| Tumor capsule formation | 66.7 | 81.4 | NS |
| Tumor capsule infiltration | 45.0 | 29.6 | NS |
| Intra-tumor septum formation | 6.7 | 6.7 | NS |
| Gross vascular or duct invasion | 23.3 | 6.2 | 0.01 |
| Microvascular invasion | 86.6 | 33.9 | 0.00 |
| Glisson's capsule invasion | 70.0 | 29.8 | 0.00 |
| Edmond-Steiner grade ³ | 47.4/52.6 | 40.7/59.3 | NS |
| Surgical factors | | | |
| Anatomical resection | 93.3 | 87.8 | NS |
| Resection margin \geq 1 cm | 66.7 | 42.3 | 0.02 |

¹Expanding growth pattern; ²Infiltrative growth pattern; ³Data in each parameter showed less than 2% missing data rate, but there was 11.5% missing rate in the Edmond-Steiner grade.

Table 3 Multivariate analysis of significant risk factors for immediate postoperative diffuse intrahepatic recurrence or multiple distant recurrence after curative resection of HCC

| Risk factors | Odds ratio | Standard error | P |
|--------------------------|------------|----------------|------|
| Microvascular invasion | 4.89 | 0.62 | 0.01 |
| AFP \geq 1 000 ng/mL | 2.98 | 0.46 | 0.02 |
| Tumor size \geq 6.5 cm | 2.98 | 0.50 | 0.03 |

tumor recurrence within 6 mo after curative resection. Among these 42 patients, 20 (47%) patients demonstrated diffuse intra-hepatic recurrence and 10 (24%) patients showed multiple systemic recurrence without evidence of intra-hepatic recurrence. Thus, the fatal recurrence group included 30 patients (11.2%, 30/269).

The mean disease-free survival time until recurrence

of HCC of the patients in the fatal recurrence group was 3.9 \pm 1.7 mo, and the mean survival time after recurrence was 6.7 \pm 6.1 mo. The remaining 12 patients who were not in the fatal recurrence group but had tumor recurrence or recurrences within 6 mo after resection had the mean disease-free survival time until recurrence at 3.4 \pm 1.8 months after surgery; however, the mean survival time after recurrence was 25.4 \pm 29 mo, and this was significantly longer when compared to the fatal recurrence group ($P < 0.05$).

Of the 269 patients, 41 (15%) patients died within 1 year of surgery. Among these 41 patients, 35 (13%, 35/269) patients died of recurrence.

The fatal recurrence group consisted of 23 males and 7 females with the mean age of 49.2 \pm 9.9 years. In the 239 control group patients, there were 178 males and 61 females with the mean age of 52.1 \pm 10.4 years, showing no significant difference of gender and age between the two groups.

Univariate analysis of patient factors between the two groups showed that there were significant differences with regard to the presence of pre-operative tumor-related symptoms ($P = 0.042$) and serum AFP levels ($P = 0.00$) (Table 1). The tumor-related symptoms included right upper quadrant pain, radiating shoulder pain, weight loss, and palpable abdominal mass. Univariate analysis showed that significant tumor and surgical risk factors between the two groups were tumor size, infiltrative growth pattern, multiple tumors, gross vascular or ductal invasion, microvascular invasion, Glisson's capsule invasion, and less than 1 cm resection margin (Table 2).

Multivariate analysis showed that there were three major risk factors for early post-operative fatal recurrence: microvascular invasion ($P = 0.01$, OR = 4.89); tumor size > 6.5 cm ($P = 0.03$, OR = 2.98); and pre-operative serum AFP levels $> 1 000$ μ g/L ($P = 0.02$, OR = 2.98) (Table 3).

Among the 269 patients, 27 patients had all the three risk factors, and of them 13 (48.1%) patients experienced actually fatal recurrence within 6 mo after curative surgery. There were 50 patients who had two risk factors, among them 11 (22%) patients experienced fatal recurrence. In addition, 71 patients were found to have only one risk

Table 4 Predictive values of risk factors for fatal recurrence after curative resection of HCC

| | <i>n</i> | Number of fatal recurrence ¹ | PPV (%) | NPV (%) | Test efficiency (%) | <i>P</i> ² |
|--|----------|---|---------|---------|---------------------|-----------------------|
| All three risk factors present | 27 | 13 | 48.1 | 92.9 | 88.5 | 0.023 |
| Only two risk factors present | 50 | 11 | 22.0 | 91.3 | 78.4 | 0.023 |
| Microvascular invasion and tumor size ≥ 6.5 cm | 26 | 6 | 23.1 | 90.1 | 83.6 | 0.027 |
| Microvascular invasion and AFP ≥ 1 000 ng/mL | 21 | 4 | 19.0 | 89.5 | 84.0 | 0.027 |
| Tumor size ≥ 6.5 cm and AFP ≥ 1 000 ng/mL | 3 | 1 | 33.3 | 89.1 | 88.5 | 0.027 |
| Only one risk factor present | 71 | 5 | 7.0 | 87.4 | 66.2 | 0.027 |
| Micro-vascular invasion | 33 | 3 | 9.1 | 88.6 | 78.8 | 0.027 |
| Tumor size ≥ 6.5 cm | 15 | 1 | 6.7 | 88.6 | 84.0 | 0.027 |
| AFP ≥ 1 000 µg/L | 23 | 1 | 4.3 | 88.2 | 81.1 | 0.027 |
| No risk factor present | 121 | 1 | 0.8 | 80.4 | 44.6 | 0.027 |
| Number of total patients | 269 | 30 | | | | |

PPV: positive predictive value; NPV: negative predictive value; ¹Number of patients with fatal recurrence within 6 mo of surgery; ²Statistical difference of PPV between patients with fatal recurrence within 6 months of surgery according to number of risk factors in each group.

factor, among whom 5 (7%) patients experienced fatal recurrence within 6 mo after surgery. Table 4 illustrates the statistical difference of positive predictive value between the different numbers of risk factors.

DISCUSSION

It has been known that risk factors which affect the pattern and timing of recurrence are different. In general, tumor factors have been considered as significant risk factors in early recurrence, while liver function factors have been reported more significant in late recurrent disease^[7-9]. Recurrence of HCC is the major risk factor for the survival of the patient after surgery. Previous studies have showed that early death within 1 year of surgery due to recurrence of the disease was approximately 10%^[7], which was in consistent with our study.

Curative treatment modalities for HCC are composed by surgical resection, liver transplantation, and percutaneous ablation^[10]. Among these modalities, surgical resection has been traditionally known to be the most effective mode of therapy for HCC^[11,12], but some serious problems, such as post-operative liver failure due to underlying cirrhosis and post-operative recurrence, still remain. To overcome these problems, alternative curative methods, such as local ablation therapy and liver transplantation, are being widely employed^[4,13].

Local cauterization therapies for HCC include injection of chemical agents, such as alcohol, and the use of heat energy, such as radiofrequency or microwave techniques. These are applicable for early HCC patients even with poor hepatic functional reserve who are not suitable for surgery. Among these alternatives, radiofrequency ablation is considered to be more efficacious compared to alcohol injection methods in terms of cellular necrosis at the tumor margins or destruction of intra-tumoral septum. But, complete response rates have been reported to be very low for tumors that are more than 5 cm in diameter, and the tumor may not be easily accessible due to its difficult location in the liver in some instances^[14,15].

Liver transplantation has been shown to result in a favorable outcome in selected HCC patients with uncompensated or compensated liver function^[4,16]. But,

in patients with advanced HCC, high rate of recurrence after OLT is a major problem of this mode of treatment. Possibly, accompanying immunosuppression may contribute to accelerating recurrence after OLT. Also, even if liver transplantation is indicated, the lack of donors and high cost of the procedure limits the wide employment of this modality.

According to a series reported by Llovet *et al*^[10], surgical resection can be a more effective modality, rather than OLT or local ablative treatment, especially in early stage HCC with good hepatic functional reserve. However, because of the limitations of the aforementioned local therapy and liver transplantation, it is a common practice to perform partial hepatectomy with curative intention in patients even with intermediate or advanced HCC, if surgery can completely remove the tumor and as long as the functional liver reserve allows the procedure in hopes of long-term survival^[11]. Recent advances in pre-operative liver function reserve assessment, surgical techniques and intra- and post-operative management have contributed to lowering the post-operative mortality rate after partial hepatectomy to below 5%. Subsequently, it is thought that hepatectomy has become safer than before and therefore, the indications for resection have been accordingly broadened. Therefore, in the practical field, it is thought that the efficacy of treatment is higher in advanced HCC than local ablation therapy or OLT.

When recurrent HCC appears immediately after partial hepatectomy for HCC, it is thought that metastases of occult cancer cells that were not detected either grossly or by imaging techniques were already present at the time of surgery. If it is possible to detect these occult micro-metastases in the pre-operative evaluation stage, then surgical resection of main tumor may be contraindicated. And it has been shown in previous studies that the liver regeneration process after partial hepatectomy may enhance the growth of occult metastases which rapidly develops into overt metastasis^[17-19]. Therefore, the effort to predict these micro-metastasis and systemic dissemination of cancer cells should not be indolent.

In this study, using multivariate analysis, we were able to observe that the characteristic risk factors in the fatal recurrence group were tumor size > 6.5 cm, serum AFP

levels > 1 000 µg/L and microvascular invasion. It has been well documented that large tumors are a risk factor for recurrence after surgery, and that larger the tumor size is the earlier the recurrences is after curative resection. One of the reasons for this has been postulated as the frequent presence of micro-metastases of tumor cells beyond the resection margins at the time of surgery. Lai *et al.*^[20] reported that intra-hepatic micro-satellite lesions are found at a greater distance from the primary tumors > 4 cm in size. In addition, intra-hepatic micro-satellites at greater distances from the primary tumor are observed frequently in HCC with multiple tumors, presence of vascular invasion, and microvascular invasion, thus making low-actual curability by partial hepatectomy. The mechanism for such intra-hepatic microscopic metastasis is due to vascular invasion, which is considered to be a risk factor for early post-operative recurrence. This has been confirmed by reports that have identified microvascular invasion or gross portal vein invasion as significant risk factors for recurrence after liver transplantation or partial hepatectomy for HCC^[9,21-23]. In previous studies, vascular invasion by the tumor has been regarded as microscopic intra-hepatic metastasis^[6,24].

The rate of recurrence is high after OLT for HCC that is above the Milan criteria. The only mechanism of the recurrence after OLT is due to vascular invasion of HCC cells that leads to extensive systemic dissemination and circulation of the cancer cells at the time of transplantation. This has been suggested as the mechanism responsible for early post-operative intra-hepatic recurrence or distant metastasis after partial hepatectomy^[25].

Previous studies have demonstrated that there is no significant difference in post-transplantation recurrence between the patients with solitary lesion of up to 6.5 cm in size and the patients with lesions of less than 5 cm (Milan criteria)^[16]. Interestingly, although the presence of HCC lesions of more than 5 cm is traditionally considered as advanced disease, our results could not show the tumor size > 5 cm to be a significant risk factor of the fatal recurrence group in the multivariate analysis. The statistical difference appeared when the lesion size of 6.5 cm was compared, indicating it as a significant risk factor. This result was in agreement with the previous data by Yao *et al.*^[16] who reported that the upper limit of HCC lesion size for transplantation was 6.5 cm, since the greater sized lesions led to significantly high rates of recurrence. These observations not only imply that when the size of tumor is greater than 6.5 cm, there is a greater possibility of systemic dissemination, but also that tumor greater than 6.5 cm may require strict control with adjuvant therapy against early post-operative recurrence as a consequence of circulating cancer cells or micro-metastatic lesion after partial hepatectomy for HCC.

Systemic adjuvant chemotherapy is the mainstay treatment modality for controlling systemic dissemination of cancer cells after surgical resection of HCC. Although many studies have failed to show clear benefits of adjuvant systemic chemotherapy for HCC in randomized control trials, Yamamoto *et al.*^[26] Suggested that post-operative adjuvant oral 5-fluorouracil may be beneficial for stage II

HCC patients with relatively favorable liver function and there are randomized control trials of immunotherapy and interferon therapy with positive results for the prevention of recurrence after resection of HCC^[27,28]. Needless to say, further more in-depth studies are required to clarify efficacy of adjuvant therapy, because advanced HCC may be potentially curable surgically, tumor recurrence from systemic dissemination of cancer cells is a frequent outcome of the disease. Therefore, in order to enhance surgical curability theoretically, it is essential to initially remove all gross disease while implementing pre- or post-operative chemotherapy or immunotherapy to control disseminated cancer cells or micro-metastasis.

Previous data have shown that high pre-operative AFP level is a significant risk factor for early post-operative recurrence^[29-31]. In this study, we also observed the similar result that serum AFP level > 1 000 µg/L is one of the significant risk factors. Also, the pre-operative serum AFP level is thought to be closely correlated with vascular invasion of tumor and dissemination of HCC cells^[25], and thus it may provide valuable prognostic information for the pre-operative evaluation.

In conclusion, the high risk patients who have two or three risk factors mentioned in this study (serum AFP > 1 000 µg/L, microvascular invasion, tumor size > 6.5 cm) should be the candidates of various adjuvant clinical trials against early post-operative fatal recurrence. Furthermore, our results may provide a control for comparing effectiveness of adjuvant clinical trial against early post-operative fatal recurrence after curative resection of high risk HCC patients.

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Detection of gelatinase B activity in serum of gastric cancer patients

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Abstract

AIM: To determine the proteolytic activity and expression of gelatinase B in serum of gastric cancer patients and their correlation with the stage of the tumor.

METHODS: Sera from 23 patients who underwent surgery for primary gastric cancer as the experimental group and from 11 as the control group were used to determine the proteolytic activity and its inhibition by EDTA and 1,10-phenanthroline. Gelatinase B activity was detected by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and SDS-PAGE zymography.

RESULTS: Proteolytic enzyme activity was increased in gastric cancer patients when compared to the control group ($P < 0.05$). The proteinases were determined to be metalloproteinases upon inhibition test with specific metalloproteinase inhibitors 1,10-phenanthroline ($P < 0.05$) and EDTA ($P < 0.01$). SDS-PAGE and SDS-PAGE zymography revealed gelatinase B (proMMP-9) activity and its molecular mass of 92 ku.

CONCLUSION: Proteinase activity is overexpressed in serum of gastric cancer patients. Gelatinase B in serum plays an important role in the progression of gastric cancer. ProMMP-9 can be used as a marker for invasiveness of gastric cancer.

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Key words: Matrix metalloproteinase-9; Gastric cancer;

Proteolytic activity; Inhibition

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INTRODUCTION

Proteolysis occurs in normal tissue but is limited in duration. A general aspect of malignant neoplasms may be an unbalance of proteolysis, which favors invasion^[1].

Tumor progression is a step-wise process. Multiple alterations in normal cells can lead to a localized tumor that can finally invade the surrounding tissues and metastasize. Tumor cell invasion involves attachment of tumor cells to the underlying basement membrane, local proteolysis and migration of tumor cells through the proteolytically modified region^[2]. Local proteolysis is facilitated by proteinases outside the tumor cells, perhaps bound to the cell surface and/or secreted from the tumor cells. Recent data suggest that proteinases inside the tumor cells also participate in local proteolysis by digesting phagocytic extracellular matrix. In order to metastasize, cells must be able to move into the vasculature (intravasation), survive in the circulation, move out of the vasculature (extravasation), invade the surrounding tissues and grow. All these steps involve interactions among tumor cells, stromal cells, invading lymphocytic cells, endothelial cells, and extracellular matrix. Proteinases expressed in these cells are believed to participate in many of these steps^[3-7].

Matrix metalloproteinases (MMPs) are extracellular enzymes capable of degrading many extracellular matrix proteins. They are classified into five groups according to their structure and substrate specificity: collagenases, gelatinases, stromelysins, matrilysins and membrane-type MMPs. There is considerable evidence that MMPs play a major role in diverse physiologic processes and pathologic processes, including aspects of embryonic development, tissue morphogenesis, wound repair, inflammatory diseases and cancer. Overexpression and activation of MMPs have been linked with a variety of diseases^[8-10].

In the matrix metalloproteinase of MMP family, including a 72 ku enzyme resembling matrix metalloproteinase-2 (MMP-2) known as gelatinase A and

a 92 ku enzyme resembling matrix metalloproteinase-9 (MMP-9) known as gelatinase B, have been demonstrated to be closely associated with several tumor systems and to invasive potential of tumor cells^[11-13]. Type IV collagenase can degrade not only interstitial matrix but also the basement membrane. Malignant ascites^[14] is the direct and prominent manifestation of advanced malignant diseases associated with invasion and metastasis of peritoneal cavity by tumor cells. In the present study, we detected the gelatinase B activity in the sera from patients with gastric cancer by gelatin zymography in order to provide the scientific basis for clinical diagnosis of gastric cancer.

MATERIALS AND METHODS

Reagents

N^ε-benzoyl-arginine p-nitroanilide hydrochloride (BAPNA), EDTA and 1,10-phenanthroline were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). The chemicals used for electrophoresis were from Merck (Darmstadt, Germany). Gelatin was purchased from Difco (Detroit, MN, USA). The mini gel electrophoresis equipment SE260 was from Hoefer Scientific Instruments (San Francisco, CA, USA).

Clinical specimens

In this study, we used 23 patients with gastric cancer as the experimental group and 11 patients as the control group. All patients with gastric cancer underwent surgery in the Institute for Digestive Diseases, Clinical Center of Serbia, from June 2002 to January 2004 and received neither chemotherapy nor radiation therapy before surgery. Of these patients, 15 (65%) were men and 8 (35%) were women with a mean age of 58 years (range: 38-75 years).

We had preoperative pathological diagnoses for all patients. Eleven patients underwent abdominal exploration or feeding jejunostomy because of liver metastases, peritoneal dissemination or malignant ascites. Twelve patients underwent radical surgery. In these patients, pathological examinations including depth of the tumor invasion, vascular invasion, lymphatic permeation, and lymph node metastasis were made according to the general rules of gastric cancer outlined by the Japanese Research Society for Gastric Cancer. In the control group, all the 11 patients were diagnosed to have groin hernia.

According to the TNM Classification System of the UICC, there were 2 stage 1, 3 stage 2, 5 stage 3, and 2 stage 4 tumors. According to their histological differentiation, there were 3 well, 4 moderately, and 5 poorly differentiated tumors. The clinicopathological features were found by reviewing all HE stained tissue sections.

Proteolytic activity

Proteolytic activity was determined using the method described by Ebeling *et al*^[15].

Metalloproteinase inhibition test

The effect of EDTA and 1,10-phenanthroline (in concentration of 5 mmol/L) on proteolytic activity of the serum was examined. The serum was incubated at 37 °C for 30 min and the remaining proteolytic activity was

determined under standard conditions.

SDS-PAGE

SDS-PAGE was performed with 75 g/L polyacrylamide gel^[16] under no reduction conditions using a solution mixture of protein markers containing ovalbumin (45 ku), bovine serum albumin (BSA, 67 ku), β-galactosidase (116 ku) and myosin (200 ku). Serum was diluted in 200 g/L sucrose to prepare the samples. The samples were analyzed by SDS-PAGE to determine the molecular mass.

SDS-PAGE zymography

Samples were analyzed by SDS-PAGE zymography according to the method of Kleiner and Stetler-Stevenson^[17] to determine the molecular mass and relative abundance of the gelatinases present. Samples were incubated for 40 min at 37 °C and electrophoreses were performed without reduction of 75 g/L polyacrylamide gels copolymerized with 0.01 g/L gelatin at 4 °C at a constant current of 15 mA. When the tracking dye at the front reached the bottom of the gel, the gel was removed and shaken gently for 45 min in 0.25 g/L Triton x-100 to remove SDS. Then the gel slabs were transferred to a bath (without Triton x-100) and washed for 20 min to remove Triton x-100. The above procedure was repeated twice at 4 °C. Then the gels were incubated and shaken for 60 h in 0.1 mol/L glycine, 50 mmol/L Tris-HCl, 5 mmol/L CaCl₂, 1 μmol/L ZnCl₂, 0.5 mol/L NaCl, pH 8.3, at 37 °C. Regions of proteolytic activity were visualized as clear zones against a blue background after 3-h staining with Coomassie brilliant blue.

MMP inhibition test on zymography

In order to verify that the clear zones resembling matrix metalloproteinase, 5 mmol/L EDTA was added into the samples before incubation to inhibit MMP activities on gelatin zymography.

Statistical analysis

Mann-Whitney test and Wilcoxon signed rank test were used for statistical analysis. $P < 0.05$ was considered statistically significant.

RESULTS

Proteolytic activity

Proteolytic activity was increased ($P < 0.05$) in gastric cancer patients compared to the control group (Tables 1 and 2). On the other hand, there was no significant correlation in proteolytic activity among the patients after radical or palliative surgery.

Metalloproteinase inhibition test

EDTA and 1,10-phenanthroline inhibited proteolytic activity on BAPNA superstrate in the sera of patients with gastric cancer. 1,10-phenanthroline ($P < 0.05$) showed less inhibition on proteolytic activity than EDTA ($P < 0.01$) (Table 1).

Detection of gelatinase B in serum

The samples of gastric cancer patients were shown on

Table 1 Proteolytic activity and inhibition of metalloproteinase activity in serum of patients with gastric cancer

| Sample | BAPNA (mU) | EDTA (mU) | Inhibition in presence of EDTA (%) | 1,10-Phen (mU) | Inhibition in presence of 1,10-phen (%) | TNM stage |
|-----------------|------------|-----------|------------------------------------|----------------|---|-----------|
| 1 | 8.6 | 1.4 | 83.7 | 0.9 | 89.5 | - |
| 2 ¹ | 3.1 | 1.7 | 45.2 | 1.6 | 51.6 | II |
| 3 ¹ | 9.0 | 2.3 | 74.4 | 1.7 | 81.1 | IV |
| 4 ¹ | 4.0 | 2.8 | 30.0 | 4.2 | - | III |
| 5 ¹ | 8.8 | 0.0 | 100.0 | 2.2 | 75.0 | IV |
| 6 | 4.5 | 5.2 | - | 1.4 | 68.9 | - |
| 7 | 0.7 | 2.1 | - | 3.5 | - | - |
| 8 | 4.9 | 5.3 | - | 8.0 | - | - |
| 9 ¹ | 7.0 | 3.3 | 52.9 | 5.7 | 18.6 | III |
| 10 | 6.2 | 0.0 | 100.0 | 3.8 | 38.7 | - |
| 11 ¹ | 2.8 | 5.6 | - | 4.2 | - | II |
| 12 ¹ | 1.7 | 1.5 | 11.8 | 6.1 | - | II |
| 13 | 6.9 | 0.6 | 91.3 | 0.7 | 89.9 | - |
| 14 | 8.0 | 0.3 | 96.2 | 1.9 | 76.2 | - |
| 15 ¹ | 5.2 | 0.1 | 98.1 | 1.5 | 71.2 | III |
| 16 ¹ | 1.8 | 0.7 | 61.1 | 2.5 | - | I |
| 17 | 0.8 | 0.7 | 12.5 | 2.3 | - | - |
| 18 | 8.8 | 1.1 | 87.5 | 5.0 | 43.2 | - |
| 19 ¹ | 4.7 | 1.9 | 59.6 | 3.6 | 23.4 | III |
| 20 ¹ | 4.8 | 1.5 | 68.8 | 1.9 | 60.4 | III |
| 21 | 5.3 | 0.0 | 100.0 | 2.4 | 54.7 | - |
| 22 | 4.1 | 0.6 | 88.7 | 2.4 | 41.7 | - |
| 23 ¹ | 1.3 | 0.5 | 61.5 | 1.1 | 15.4 | I |

¹Radical surgery.

SDS-PAGE Coomassie brilliant blue staining bands at the mass position of 92 ku. The protein molecular mass of 92 ku was detected in 82% of patients with gastric cancer. Molecular mass of 92 ku indicated MMP-9 protein (Figure 1). In the control group, MMP-9 protein was not detected.

The gelatinase B activity was detected by SDS-PAGE zymography as the clear bands against the blue background (Figure 1) in the sera of gastric cancer patients. There were no clear bands in the control group. The clear bands detected by gelatin zymography were characterized by the activity of gelatinases A (72 ku) and B (92 ku). The reaction was positive for band migrating at approximately 220 and 92 ku and for bands at 200 and 116 ku in some samples. The 220-ku band was strongly positive for gelatinase B, suggestive of homodimer. The 200- and 116-ku bands were interpreted as proMMP-9/TIMP-1 complexes.

Metalloproteinase inhibition test by zymography

Gelatinase B activity in the serum of gastric cancer patients was inhibited by EDTA.

DISCUSSION

Proteolytic activity in the sera of patients with gastric cancer was higher than that in the control group, indicating that proteolysis can be degraded by ECM^[18-20]. For the occurrence of metastasis, tumor cells must repeatedly cross over the basement membrane barrier, a process for which

Table 2 Proteolytic activity and inhibition of metalloproteinase activity in serum of control group

| Control | BAPNA (mU) | EDTA (mU) | Inhibition in presence of EDTA (%) | 1,10-Phen (mU) | Inhibition in presence of EDTA (%) |
|---------|------------|-----------|------------------------------------|----------------|------------------------------------|
| 1 | 1.3 | 4.3 | - | 0.4 | 69.2 |
| 2 | 0.7 | 3.2 | - | 4.3 | - |
| 3 | 0.4 | 2.3 | - | 1.7 | - |
| 4 | 4.4 | 6.8 | - | 0.9 | 79.5 |
| 5 | 0.3 | 1.3 | - | 1.7 | - |
| 6 | 2.2 | 0.9 | 59.1 | - | - |
| 7 | 0.9 | 1.6 | - | 2.5 | - |
| 8 | 1.0 | 0.0 | 100.0 | 3.2 | - |
| 9 | 0.3 | 0.9 | - | 2.6 | - |
| 10 | 0.3 | 1.1 | - | 2.7 | - |
| 11 | 1.7 | 0.0 | 100.0 | 0.0 | 100.0 |

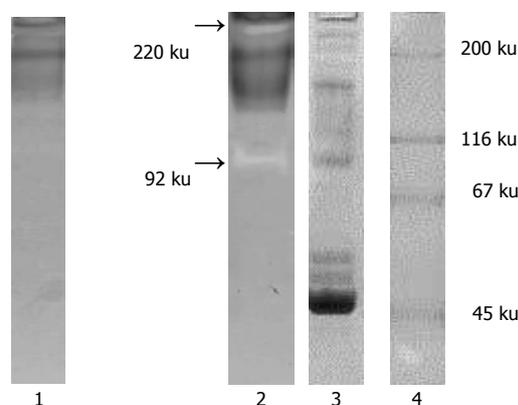


Figure 1 Results of SDS-PAGE and SDS-PAGE zymography. 1: Serum of gastric cancer patients with EDTA inhibitor; 2: serum of control group; 3: molecular mass determination; 4: Protein marker mixture.

proteolysis of ECM components is required^[21,22]. Some of the proteins associated with invasion and metastases of tumors are produced by tumor cells. Then, the proteins (whole or fragments) may accumulate in blood or urine of patients.

According to the inhibition test with EDTA and 1,10-phenanthroline, proteinases are found to be metalloproteinases and the inhibition is an additional biochemical parameter for correlation of proteolytic activity and gastric cancer. Increased levels of metalloproteinase have been implicated in the invasive potential of tumors^[23-26]. These results suggest that overexpression of metalloproteinases in the serum plays an important role in the progression of gastric cancer.

Overexpression of type IV collagenase has been demonstrated in a variety of cancers including colorectal cancer^[27], gastric cancer^[28], and breast cancer. There is evidence that type IV collagenase activity or concentration is increased in the plasma of patients with advanced carcinoma^[29-33]. It was reported that type IV collagenase activity is increased in urine^[34] and ascites^[14] of cancer patients.

In the present study, we initially measured the gelatinase B activities in the serum of gastric cancer patients. The

results demonstrated that proteolytically active proMMP-9 was significantly associated with cancer. Proteolytic activity was shown in tumor patients. On the basis of molecular size and inhibition by EDTA, the bands were respectively interpreted as proMMP-9 (92 ku) and its putative dimer 220 ku and proMMP-9/TIMP-1 complexes (200 and 116 ku). The activated form of gelatinase B (83 ku) was not detected in the serum of cancer patients. Gelatinase A activity was not detected in the serum of gastric cancer patients. The gelatinases, particularly gelatinase A, seem to be important in the initial stage of tumor invasion^[12] as they degrade the components of the basement membrane, while other MMPs contribute to the later stages of tumor invasion^[35]. In some reports^[36], gelatinase B in gastric carcinoma is positively correlated with the existence of vessel permeation, lymph node metastasis or the depth of tumor invasion.

In conclusion, gelatinase B protein may serve as a marker for invasiveness and metastasis of gastric cancer^[37-39]. ProMMP-9 can be used for the detection of primary or recurrent cancer and for the estimation of tumor extent.

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RAPID COMMUNICATION

Changes of plasma fasting carnitine ester profile in patients with ulcerative colitis

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Abstract

AIM: To determine the plasma carnitine ester profile in adult patients with ulcerative colitis (UC) and compared with healthy control subjects.

METHOD: Using ESI triple quadrupole tandem mass spectrometry, the carnitine ester profile was measured in 44 patients with UC and 44 age- and sex-matched healthy controls.

RESULTS: There was no significant difference in the fasting free carnitine level between the patients with UC and the healthy controls. The fasting propionyl- (0.331 ± 0.019 vs 0.392 ± 0.017 $\mu\text{mol/L}$), butyryl- (0.219 ± 0.014 vs 0.265 ± 0.012), and isovalerylcarnitine (0.111 ± 0.008 vs 0.134 ± 0.008) levels were decreased in the UC patients. By contrast, the level of octanoyl- (0.147 ± 0.009 vs 0.114 ± 0.008), decanoyl- (0.180 ± 0.012 vs 0.137 ± 0.008), myristoyl- (0.048 ± 0.003 vs 0.039 ± 0.003), palmitoyl- (0.128 ± 0.006 vs 0.109 ± 0.004), palmitoleyl- (0.042 ± 0.003 vs 0.031 ± 0.002) and oleylcarnitine (0.183 ± 0.007 vs 0.163 ± 0.007 ; $P < 0.05$ in all comparisons) were increased in the patients with UC.

CONCLUSION: Our data suggest selective involvement of the carnitine esters in UC patients, probably due to their altered metabolism.

INTRODUCTION

Ulcerative colitis (UC) is a disorder of the idiopathic and chronic inflammation of the colonic mucosa. The etiology and the pathogenesis of the disease are yet unknown; a classic study on isolated colonic epithelial cells demonstrated decreased utilization of n-butyrate. Since the major energy sources of the epithelial cells of the distal colon are the short-chain fatty acids (SCFAs), these cells are able to metabolize other fuels, such as glucose and glutamine, only at a much lower rate^[1]. SCFAs are generated from carbohydrates by bacterial degradation and they are readily absorbed by the colon and represent energy fuels for the colonocytes and other tissues, such as the skeletal muscle^[2,3]. Patients with distal UC may have increased or moderately decreased stool SCFA concentrations, reflecting their altered absorption^[4,5]. UC can, therefore, be regarded essentially as an SCFA oxidation failure-associated disease, where the energy deficiency is a primary event in the development of the disease^[1].

L-carnitine plays an essential role in the energy metabolism, since it enables the transport of activated long-chain fatty acids (LCFA) as carnitine esters across the inner mitochondrial membrane. Moreover, it is able to form esters with several medium- and SCFAs of both endogenous and exogenous origins^[6,7]. The impact of altered SCFA metabolism in UC prompted us to study the circulating carnitine ester profile in the UC patients.

MATERIALS AND METHODS

Patients

We examined 44 patients with UC (25 males, 19 females,

Table 1 Some major clinical and laboratory parameters in patients with ulcerative colitis and control subjects (mean \pm SE)

| Parameters | UC patients, <i>n</i> = 44 | Controls, <i>n</i> = 44 |
|-----------------------------------|----------------------------|-------------------------|
| Females/males | 19/25 | 24/20 |
| CRP (mg/L) | 12.2 \pm 4.5 | 2.6 \pm 0.5 |
| Albumin (g/L) | 44.6 \pm 0.7 | 50.2 \pm 0.8 |
| Iron (μ mol/L) | 16.1 \pm 1.2 | 23.7 \pm 1.6 |
| Hb (g/dL) | 131.3 \pm 2.5 | 159 \pm 1.2 |
| MCV (L) | 86.2 \pm 1.2 | 94.8 \pm 2.5 |
| WBC (G/L) | 7.6 \pm 0.4 | 9.2 \pm 0.6 |
| BMI (kg/m ²) | 24.6 \pm 0.6 | 24.1 \pm 0.5 |
| PLT (G/L) | 298.5 \pm 13.5 | 228.3 \pm 10.4 |
| Both ileum and colon localization | 5/44 (11.4%) | |
| Rectosigmoid localization only | 8/44 (18.2%) | |
| Colon localization | 31/44 (70.4%) | |

mean age: 39.7 years, range: 17-65 years), and 44 carefully selected clinically healthy age-, sex-, weight-, and height-matched control subjects (20 males, 24 females, mean age: 37.0 years, range: 23-60 years). The control subjects did not receive any drug medication, while the UC patients were treated with either sulfasalazine or 5-aminosalicylic acid. We assumed that these drugs do not have any influence on the carnitine status since there were no such data available in the literature.

Diagnosis of the disease relied upon the history of the patients, clinical symptoms, negative stool examination for bacteria and parasites, and histologic results of rectal and/or colonic biopsy. Exclusion criteria in both groups were as follows: secondary causes of colonic disease, systemic diseases, any malformations, evidence of intestinal bacterial infection, history or evidence of any inherited metabolic disease, hepatic or renal disease, and pregnancy. (Table 1) shows the clinical parameters of the UC patients.

The clinical and laboratory data were the results of measurements performed from sample aliquots of blood collected after an overnight fasting precisely between 08:00 a.m. and 08:30 a.m., both in the UC patients and in the healthy control subjects. This strict post-alimental time scheduling was introduced to prevent the diet or fasting time-induced dynamic changes of carnitine esters in the circulation^[8].

Informed consent was obtained from each participant of the study and the study design was approved by the Departmental Ethics Committee.

Methods

Plasma albumin, iron, and C-reactive protein levels were determined by routine methods. The hemoglobin (Hb), mean corpuscular volume (MCV), white blood cells (WBC) and platelet (PLT) were measured by automated analysis (sysmex XE 2100, Japan). The body mass index (BMI) was calculated as body weight/height² (in kg/m²).

Acylcarnitines were measured after derivatization as butyl esters using isotope dilution mass spectrometry method in a Micromass Quattro Ultima ESI triple-quadrupole mass spectrometer. The procedure was principally the method described previously^[9]. Essentially,

10 μ L of plasma was spotted and dried onto a filter paper and prepared by extraction with 200 μ L of methanol containing internal deuterium-labeled standards (0.76 μ mol/L [²H₃]-free carnitine, 0.04 μ mol/L [²H₃]-propionylcarnitine, 0.04 μ mol/L [²H₃]-octanoylcarnitine and 0.08 μ mol/L [²H₃]-palmitoylcarnitine). After 20 min of agitation, the supernatant was evaporated to dryness under nitrogen at 40 °C and then 100 μ L of 3 mol/L butanolic HCl was added. The solution was incubated at 65 °C for 15 min and evaporated to dryness again under nitrogen at 40 °C and re-dissolved in 100 μ L of the mobile phase (acetonitrile:water 80:20). A total of 10 μ L of sample aliquots were introduced to the ESI cone by using Waters 2795 HPLC system. The free carnitine and all acylcarnitines were determined by ESI-MS/MS analysis using positive precursor ion scan of m/z 85; scan range was 200-550 m/z . The capillary voltage was 2.50 kV, while the cone voltage was 55 V, and the collision energy was 25 eV. The flow rate was 100 μ L/min and the total analysis time was 4 min per sample. Each sample was measured in triplicates starting with the injection step, and the results were the means of the three determinations.

Statistical analysis

Student's *t* test for unpaired samples was used for the statistical analysis. The values were expressed as mean \pm SE, in three decimals for the carnitine esters with respect to the low levels in the case of the long-chain carnitine esters. $P < 0.05$ was considered statistically significant.

RESULTS

The plasma circulating carnitine ester profiles are shown in Table 2. The plasma level of free carnitine and acetyl carnitine did not differ between the UC patients and the controls. By contrast, significant decreases were observed in fasting propionyl-, butyryl-, and isovaleryl carnitine ester levels in UC patients as compared with the controls. The level of total short-chain carnitine esters was markedly lower in the patients with UC (9.855 \pm 0.094 μ mol/L) than in the healthy controls (11.003 \pm 0.100 μ mol/L, $P < 0.01$).

The levels of octanoyl-, and decanoylcarnitine were decreased in the healthy subjects. The levels of total medium-chain acylcarnitines were obviously higher in the UC patients (0.629 \pm 0.007 μ mol/L) than in the control subjects (0.548 \pm 0.007 μ mol/L, $P < 0.01$).

In the long-chain acylcarnitine group, the plasma levels of myristoyl-, palmitoyl-, palmitoleyl- and oleylcarnitine were significantly decreased in the healthy group. The levels of total long-chain carnitine esters were markedly higher in the patients with UC (0.596 \pm 0.005 μ mol/L) than in the controls (0.515 \pm 0.009 μ mol/L, $P < 0.01$).

In addition, the level of total carnitine esters was significantly decreased in the UC patients (11.080 \pm 0.035 μ mol/L) as compared with the healthy controls (12.066 \pm 0.037 μ mol/L, $P < 0.01$).

DISCUSSION

Carnitine [β -hydroxy- γ (trimethylamino) butyric acid]

Table 2 Plasma carnitine ester profiles in ulcerative colitis patients and controls ($\mu\text{mol/L}$, mean \pm SE)

| | UC patients, n = 44 | Controls, n = 44 |
|---------------------------------------|--------------------------------|--------------------|
| Free carnitine (C0) | 31.595 \pm 1.454 | 31.431 \pm 1.042 |
| Short-chain acylcarnitines | | |
| Acetylcarnitine (C2) | 9.164 \pm 0.426 | 10.179 \pm 0.461 |
| Propionylcarnitine (C3) | 0.331 \pm 0.019 ^a | 0.392 \pm 0.017 |
| Butyrylcarnitine (C4) | 0.219 \pm 0.014 ^a | 0.265 \pm 0.012 |
| Isovalerylcarnitine (C5) | 0.111 \pm 0.008 ^a | 0.134 \pm 0.008 |
| Tiglylcarnitine (C5:1) | 0.030 \pm 0.004 | 0.033 \pm 0.002 |
| Medium-chain acylcarnitines | | |
| Hexanoylcarnitine (C6) | 0.071 \pm 0.006 | 0.081 \pm 0.005 |
| Octenoylcarnitine (C8) | 0.147 \pm 0.009 ^a | 0.114 \pm 0.008 |
| Octenoylcarnitine (C8:1) | 0.064 \pm 0.005 | 0.062 \pm 0.007 |
| Decanoylcarnitine (C10) | 0.180 \pm 0.012 ^a | 0.137 \pm 0.008 |
| Cecenoylcarnitine (C10:1) | 0.113 \pm 0.006 | 0.104 \pm 0.008 |
| Lauroylcarnitine (C12) | 0.054 \pm 0.003 | 0.050 \pm 0.003 |
| Long-chain acylcarnitines | | |
| Myristoylcarnitine (C14) | 0.026 \pm 0.001 | 0.024 \pm 0.001 |
| Myristoleylcarnitine (C14:1) | 0.048 \pm 0.003 ^a | 0.039 \pm 0.003 |
| Palmitoylcarnitine (C16) | 0.128 \pm 0.006 ^a | 0.109 \pm 0.004 |
| Palmitoylcarnitine (C16:1) | 0.042 \pm 0.003 ^a | 0.031 \pm 0.002 |
| Stearoylcarnitine (C18) | 0.085 \pm 0.003 | 0.079 \pm 0.003 |
| Oleylcarnitine (C18:1) | 0.183 \pm 0.007 ^a | 0.163 \pm 0.007 |
| Hydroxymyristoylcarnitine (C14OH) | 0.007 \pm 0.001 | 0.006 \pm 0.001 |
| Hydroxypalmitoylcarnitine (C16OH) | 0.026 \pm 0.002 | 0.023 \pm 0.001 |
| Hydroxypalmitoleylcarnitine (C16:1OH) | 0.033 \pm 0.002 | 0.029 \pm 0.002 |
| Hydroxyoleylcarnitine (C18:1OH) | 0.018 \pm 0.002 ^a | 0.012 \pm 0.001 |

^aP < 0.05 vs controls.

is known as a carrier for transporting activated LCFA into the mitochondrial matrix for β -oxidation. With this function the L-carnitine plays an essential role in the energy metabolism^[6]. Moreover, the carnitine molecule is able to form esters with several medium- and short-chain fatty acids of both endogenous and exogenous origins^[6,7]. The circulating carnitine ester spectrum can reflect affected cellular metabolism of the short-, medium-, and long-chain fatty acids. Therefore, the monitoring of the carnitine ester composition is a widespread tool for the diagnosis of several inborn errors of metabolism. Besides the complete metabolic blockage caused by the inherited lack of enzyme activities, influences on carnitine ester spectra may be the consequence of only partially affected flux of metabolites via the carnitine acyltransferases.

In the present study, significant decrease was found in the fasting plasma levels of propionyl-, butyryl-, and isovalerylcarnitine esters, leading to the decrease of SCFA carnitine esters. Although the pathogenesis of UC is still unknown, a widely accepted hypothesis focuses on the pivotal role of the diminished availability of SCFAs for the enteral cells. Normally, SCFAs are rapidly absorbed from the colon and have many properties, as they represent an energy source for colonocytes and if they are exported to other tissues. Moreover, they affect lipid metabolism, colonic mucosal blood flow, motility, and mucus secretion^[2]. In the normal case, the major energy source of the epithelial cells of the distal colon derives from the metabolism of the SCFAs^[10], which is impaired in UC^[1]. In addition to the SCFA metabolism, the influenced coenzyme A esterification has been reported to be associated with UC^[11]. In the cells, the fatty acyl

moieties are transferred from coenzyme A to the beta hydroxyl group of the carnitine via the short-, medium-, and long-chain carnitine acyltransferases^[6]. These events separately or in combination, can explain the decrease of the circulating SCFA carnitine esters.

The circulating plasma carnitine profile is determined by the balance of the release and uptake mechanisms. Carnitine releases into the circulation by the liver primarily as acylcarnitine^[12]. While in the hepatic vein, the ester proportion is relatively high, approximately half of the total carnitine is esterified. The actual ester pattern detected in the peripheral blood is a result of the uptake/release action of the peripheral tissues; and in a peripheral venous blood, much less (approximately 1/3-1/4 of the total carnitine) is esterified. Whereas, the decrease of the SCFA carnitine esters found in the UC patients could be explained as discussed earlier. Based on the current knowledge, it is much more difficult due to the limited nature of the data, to explain the opposite change of the medium- or long-chain carnitine esters. Only a few studies are available reporting alterations of fatty acid metabolism. However, the data are inconsistent, but suggest the involvement of LCFA metabolism in UC^[13-15]. Further studies are required to clarify these issues.

After the positive results on topical SCFA treatment in UC^[16], Gasbarrini *et al*^[3] studied propionyl-L-carnitine administration as rectal irrigation and found that improved clinical picture and histological status of the bowel are improved. In the light of the current findings, the likely decreased tissue reserves could be corrected by administration of the drug and the positive outcome could be a consequence of the successful replacement therapy. Whether the already known beneficial therapeutic effects of special LCFA containing or supplemented with diets^[14,15,17-20] are due to at least in part, a similar replacement phenomenon, also remains to be elucidated.

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RAPID COMMUNICATION

Effect of percutaneous endoscopic gastrostomy on gastroesophageal reflux in mechanically-ventilated patients

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Abstract

AIM: To investigate the effect of percutaneous endoscopic gastrostomy (PEG) on gastroesophageal reflux (GER) in mechanically-ventilated patients.

METHODS: In a prospective, randomized, controlled study 36 patients with recurrent or persistent ventilator-associated pneumonia (VAP) and GER > 6% were divided into PEG group ($n = 16$) or non-PEG group ($n = 20$). Another 11 ventilated patients without reflux (GER < 3%) served as control group. Esophageal pH-metry was performed by the "pull through" method at baseline, 2 and 7 d after PEG. Patients were strictly followed up for semi-recumbent position and control of gastric nutrient residue.

RESULTS: A significant decrease of median (range) reflux was observed in PEG group from 7.8 (6.2 - 15.6) at baseline to 2.7 (0 - 10.4) on d 7 post-gastrostomy ($P < 0.01$), while the reflux increased from 9 (6.2 - 22) to 10.8 (6.3 - 36.6) ($P < 0.01$) in non-PEG group. A significant correlation between GER (%) and the stay of nasogastric tube was detected ($r = 0.56$, $P < 0.01$).

CONCLUSION: Gastrostomy when combined with semi-recumbent position and absence of nutrient gastric residue reduces the gastroesophageal reflux in ventilated patients.

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Key words: Nasogastric tube; Gastroesophageal reflux; Semi-recumbency; Gastric residue; Percutaneous endo-

INTRODUCTION

Life threatening nosocomial pneumonia secondary to aspiration of gastric contents is frequent in intubated mechanically-ventilated patients^[1,2]. A number of causes have been implicated in the development of ventilator-associated pneumonia (VAP) during mechanical ventilation, namely the oropharyngeal colonization^[2], body position^[3], nasogastric tube (NGT)^[4], and its size^[5]. Supine position and the length of patient's permanence in this position are other potential risk factors for aspiration^[3]. Though the semi-recumbent position reduces the risk of pulmonary aspiration, gastroesophageal reflux (GER) is still possible^[6,7]. However, GER occurs with a significantly higher incidence in semi-recumbent mechanically-ventilated patients with NGT than without (74% vs 35%)^[8]. According to other studies^[9,10], large-bore tubes do not cause more reflux than small-bore ones.

If the duration of nasogastric intubation correlates with the degree of GER, NGT removal after gastrostomy should normally decrease both GER and aspiration pneumonia rates. Percutaneous endoscopic gastrostomy (PEG), however, is considered neither as a non-pharmacological measure for the prevention of VAP, nor as an adjunctive measure to its treatment because it elicits an increase in GER, aspiration, and incidence of pneumonia, at least in the early post-gastrostomy period^[8,11]. Nevertheless, in these studies the body position was not specified and the gastric content was not controlled after gastrostomy.

The aim of the present study was to investigate if long-standing presence of NGT for feeding is associated with increased incidence of GER and if PEG combined with semi-recumbent position and avoidance of gastric nutrient retention lead to decreased incidence of GER in mechanically-ventilated patients.

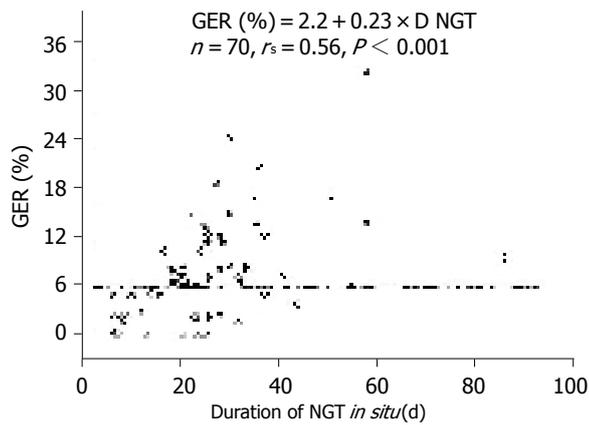


Figure 1 Correlation between duration of NGT and mechanical ventilation standing period to the GER rate measured in 70 patients.

Table 1 Characteristics of patients in the study [median (range)]

| | PEG (n=16) | Non-PEG (n=20) | Control (n=11) | P |
|------------------------------|------------|----------------|----------------|----|
| Age | 53 (20-82) | 58 (25-85) | 56 (34-76) | NS |
| Sex (M/F) | 10/6 | 12/8 | 7/4 | NS |
| Weight (kg) | 75 (55-85) | 79 (56-95) | 83(68-90) | NS |
| APACHE II | 17 (12-23) | 17 (9-28) | 15 (5-26) | |
| Primary disease | | | | |
| Head injury | 7 | 5 | 1 | |
| Spinal cord injury | 3 | 4 | - | |
| Multiple trauma | 5 | 8 | 3 | NS |
| Stroke | 4 | 2 | 5 | |
| COPD | 5 | 4 | 3 | |
| Post-operative resuscitation | 4 | 3 | - | |
| Days of MV and NGT | 25 (19-36) | 27 (17-56) | 24 (12-32) | NS |
| Days of VAP before study | 14 (8-19) | 13 (4-36) | - | NS |

MATERIALS AND METHODS

Pilot study

Simultaneous measurement of gastric and esophageal pH was performed during a 24-h period^[12] in 23 adult intensive care patients, who were mechanically-ventilated and had a NGT in place for a duration of 3-90 d. Exclusion criteria from the study included unstable hemodynamic state, administration of morphine, atropine, theophylline, barbiturates, and cisapride, and a past history of GER or hiatal hernia. GER was expressed as percentage of the time when the esophageal pH was less than 4 in a given 24-h period of time GER (%). Values of GER less than 3% were considered as normal. In these 23 patients, there was a positive correlation of the duration of nasogastric intubation in mechanically-ventilated patients to the degree of GER (%) ($r=0.78$, $P<0.01$). GER mainly occurred after a 10-d period of NGT and mechanical ventilation. Based on these results, only patients who were on mechanical ventilation with NGT for more than 10 d were enrolled in the main study.

Main study and patient selection

The institutional review board approved the study protocol and informed consent was obtained from patient'

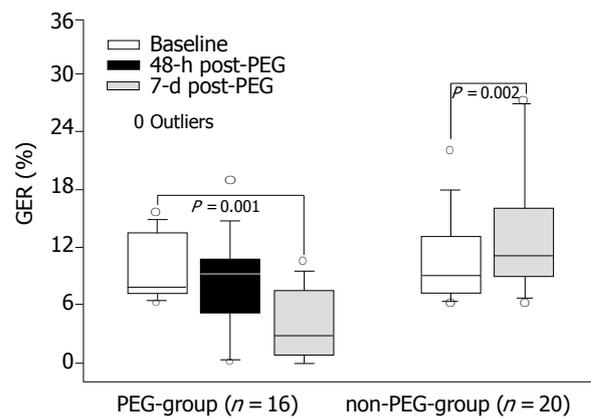


Figure 2 Variation of the median GER (%) together with 10%, 25%, 75%, and 90% from pH-metries originated from the sensor located at 20 cm proximally to the lower esophageal sphincter. The symbol in the 48-h period is lacking in patients of non-PEG-group since no pH-metry was performed in that period in this group of patients.

s kin in each case. Over a 28-mo period, 39 consecutive mechanically-ventilated patients with NGT in place, suffering from persistent or recurrent VAP and presenting a GER above 6% constituted the study population. The diagnosis of persistent or recurrent VAP was established according to the criteria previously described^[13,14]. The exclusion criteria mentioned in the pilot study were maintained. Nineteen patients were randomly allocated to receive PEG, but three among them were excluded because of hiatal hernia (2 cases) and intestinal bloating (1 case). Finally, 16 patients received PEG (PEG group) and 20 did not (non-PEG group). In the non-PEG group patients the eventual presence of hiatal hernia was possibly missed, since no endoscopy was performed for PEG placement. Twelve patients in the PEG group and 15 patients in the non-PEG group presented persistent pneumonia. The rest of the patients in both groups had recurrent pneumonia. Eleven mechanically-ventilated patients with acute respiratory failure and NGT for more than 15 d and GER lower than 3% were used as controls. Patients enrolled in the study had comparable severity scores of VAP or acute respiratory failure, radiographic scores and ventilation parameters (data not shown). The characteristics of patients are presented in (Table 1).

Gastrostomy was performed using the pull through (Ponsky) technique^[15] after feeding was stopped for 24 h. Patients of all groups were on continuous drip NGT or PEG-feeding at 60 mL/h with a polymeric diet of energy content of 1 000 kcal/L. Thereafter, they were placed in a semi-recumbent position (30°) and the volume of the gastric nutrient residue was measured with a syringe at 8-h intervals. If the nutrient volume exceeded 200 mL, feeding was withheld and restarted when the volume decreased. These two measures were followed for the subsequent 20-d period during which conventional treatment for pneumonia was applied.

All pH-metries were performed on a 24 h basis. Baseline pH-metries and those performed on d 7 in non-PEG patients were carried out as follows: with the patient in semi-recumbent position a single crystal antimony multi use electrode was used which disposes three sensors

located at the tip, 15 and 30 cm, respectively connected to a portable recorder (Digitrapper Mk III, Synectics Medical AB, Stockholm, Sweden). The electrode had a diameter of 2.1 mm at the level of the sensors and was attached via an adhesive material to a new 14 bore NGT so that the distal pH-meter sensor corresponded at 10 cm proximally to the tip of the NGT. In this way, reflux associated with the presence of NGT could be detected. An *in vitro* calibration of the whole system was carried out with buffer solutions of pH 1 and pH 7 before each pH-metry.

After ordinary NGT removal, the new NGT with the sensor probe attached was introduced via the nose into the stomach until acid pH was recorded with the distal and middle sensors as previously described^[12]. The electrode was then slowly withdrawn until the middle sensor channel detected a sudden pH change from acid (<4) to above 5. The electrode was then withdrawn for further 5 cm. In this way, the distal sensor was located into the stomach and the middle and proximal sensors were located at 5 and 20 cm above the lower esophageal sphincter, respectively. The correct positioning of the electrode was verified at the end of pH-metry and before its withdrawal by a chest x-ray. The recording device measured pH values every 4 s and stored the mean of 20 values every 80 s.

Patients received sucralfate, 2 g twice daily via NGT at least 72 h before the study for gastric mucosa protection. Antacids, H-2 blocking agents or omeprazole that could interfere with pH neutralization were not used and for the same reason feeding was stopped 6 h before and during the period of pH-metry. Patients were not sedated nor paralyzed.

In PEG group patients, two additional pH-metries were carried out: the first at an early (48 h) post-PEG period and the second at a late (7 d) post-PEG period. The first pH-metry investigated the described increased incidence of reflux and/or aspiration at that period^[8]. The second pH-metry also performed in the non-PEG group of patients was carried out in order to estimate and evaluate the effectiveness of PEG on reflux. These additional pH-metries were performed in the same manner with the following modifications: a two-sensor probe was used and positioned in such a way that the distal and proximal sensors were located at 5 and 20 cm over the lower esophageal sphincter, respectively. With the two-sensor probe the lower esophageal sphincter was free from the presence of any catheter. Patients were fed through PEG and the degree of reflux was assessed in the absence of NGT.

PEG was arbitrarily considered effective if within a 7-d post PEG period the GER (%) decreased by more than 60% compared to the pre-PEG value? PEG and non-PEG patients were followed up for 20 d for pneumonia healing and all patients for weaning from mechanical ventilation and intensive care discharge.

Statistical analysis

To evaluate the differences in GER (%) between the three study periods (pre-PEG *vs* early and late post-PEG) in each group, Wilcoxon's signed-rank test was used. To assess the changes in the number of days of mechanical ventilation and NGT standing, pneumonia occurrence

before PEG, weaning after PEG, days from PEG to discharge between the two groups, Wilcoxon's rank sum test was performed. Spearman's *r* was used to detect if there was any correlation between the standing-period of NGT and GER (%). Statistical significance was defined as a *P* value of 0.05 or less.

RESULTS

The correlation between GER (%) and duration of NGT permanence in the 23 patients from the pilot study along with the values obtained from the first pH-metry in the 47 patients of the main study is shown in Figure 1. After 20 d of NGT *in situ*, 38 out of 50 patients (76%) presented a reflux rate of above 6% while all the 14 patients with NGT *in situ* for less than 15 d had a reflux rate of less than 6%.

The pH-metry from the lower esophageal sensor consistently recorded 15-20% higher GER than that from the upper sensor. However, since the importance of reflux detection in the upper part of esophagus is greater in relation to aspiration, only data from the upper sensor were presented. The median (range) GER (%) in the PEG group was 7.8 (6.2-15.6) at baseline. Forty-eight hours post-PEG, though there was no significant change in the median value [8.7 (0.1-19)], an increase in GER (%) was observed in 5 out of 16 patients. On d 7, post-PEG, GER (%) decreased to 2.7 (0-10.4) (*P*<0.01). In contrast, in the non-PEG group, GER (%) increased from 9 (6.2-22) at baseline to 10.8 (6.3-36.6) on d 7 (*P*<0.01) (Figure 2).

In the following 20-d period, the weaned, discharged, and died patients were respectively 11 and 4 (*P*=0.006), 10 and 2 (*P*<0.01), and 3 and 5 in PEG and non-PEG groups. The respective values for patients in the control group were 8, 8, and 1. The outcome of the PEG-group was similar to that of the control group.

DISCUSSION

This study showed that the degree of GER correlated with the duration of NGT *in situ*. Removal of NGT and feeding through PEG with the patients in semi-recumbent position along with the volume control of the nutrient gastric residue resulted in a decrease or even elimination of the reflux in almost two-thirds of the patients. Additionally, PEG seemed to exert a favorable effect on pneumonia healing rate, weaning period, and patient discharge from ICU.

There are several reasons for frequent GER occurrence in the critically ill patients. Drugs depressing the function of lower esophageal sphincter and/or delaying gastric emptying, such as morphine, atropine, theophylline, and barbiturates, are frequently administered in the critically ill patients^[16]. The presence of NGT is an important cause of reflux, since it may induce lower esophageal sphincter relaxation^[17]. In patients undergoing elective laparotomy and nasogastric intubation, a significant increase in the mean number of reflux episodes has been observed during the perioperative period in contrast to those without NGT (137 *vs* 8, *P*<0.01)^[18]. The mean lower esophageal sphincter pressures are also lower in the same patients, and reflux occurs within 24 h after NGT insertion at the

induction of anesthesia. Similarly, in patients undergoing cardiac surgery with simultaneous esophageal and tracheal pH-metry, reflux is seen more frequently in those with a NGT in place than in those without ($P < 0.001$)^[19].

The above observations lead to the notion that NGT removal after PEG should theoretically eliminate or decrease the incidence of GER. However, data in the literature paradoxically support the opposite. There is evidence that PEG or percutaneous endoscopic jejunostomy provides no benefit in terms of reflux and/or incidence of aspiration pneumonia^[8,11,19-23]. In a study of 64 patients, 9 developed aspiration pneumonia within 3 d of the procedure and four died^[8]. Aspiration occurred in 11% of 79 patients with either neurologic disorders or cancer, whose PEG or percutaneous endoscopic jejunostomy was performed^[11]. In another study comprising 20 malnourished patients, aspiration was the most common adverse event following percutaneous endoscopic jejunostomy, accounting for 50% of deaths^[20]. Similarly, GER seems to be a frequent disorder following PEG in children^[21-23]. Yet, in 58 neurologically disabled patients who had clinical evidence of aspiration pneumonia, 17 demonstrated pneumonia after gastrostomy^[24]. As a result of the negative outcomes, the use of PEG has declined during recent years. However, in all these studies the body position following PEG was not reported and the volume of gastric nutrient residue was not controlled.

The positive results found in our PEG group compared to those reported in the literature have to be attributed to the volume control of the nutrient gastric residue and the semi-recumbent position during the whole 20-observation period. The semi-recumbent position has been previously shown to reduce aspiration of gastric contents^[6]. Gastric distension is also an important cause of lower esophageal sphincter transient relaxation, thus permitting GER^[25]. It seems that owing to gravity, semi-recumbency, and avoidance of gastric retention prevent reflux of gastric juice into the esophagus.

The results of the present study describe the pathophysiological sequence of PEG-induced lower esophageal sphincter restoration. In 11/16 patients in the PEG group, the reflux rate did not decrease during the first 48 post-PEG hours. Moreover, in 5 of them GER (%) increased in this period. These results indicate that "PEG was arbitrarily considered effective if within a 7-day post PEG period the GER (%) decreased by more than 60% compared to the pre-PEG value". Thus, a period of at least 7 d seems to be required after NGT removal before lower esophageal sphincter returns to its normal function. The same findings also provide an explanation for the high incidence of aspiration pneumonia encountered during the early post-PEG period^[7,8,17,18].

The role of GER in the pathogenesis of VAP is still controversial^[26-28]. All patients in the present study suffering from persistent or recurrent pneumonia were severely ill and failed to respond to the usual therapeutic measures. However, reflux was eliminated in 10 out of 16 patients undergoing PEG who weaned from the respirator and discharged from ICU within the 20-d period of observation. In contrast, among the 20 non-PEG patients reflux increased through time and only 4 and 2 patients

were weaned and discharged, respectively. The curative rate of PEG-group was similar to that of control group with the GER being lower than 3%. Among the 11 patients with GER < 3% only one presented VAP. Therefore, it is concluded that GER is implicated in the pathogenesis of VAP and the elimination of reflux results in a more favorable outcome, possibly because the repetitive instillation of infective materials to the trachea that occurs during reflux, is halted.

In conclusion, NGT presence seems to promote reflux of gastric contents, resulting in aspiration and/or pneumonia. The NGT replacement by PEG combined with semi-recumbent position and control of gastric residue can decrease GER in the majority of patients. However, there is persistence of GER and aspiration in some patients, which may be due to the functional alteration of the lower esophageal sphincter rather than ineffectiveness of PEG *per se*. Gastrostomy combined with semi-recumbency and control of gastric residue should be taken into consideration for the effective management and prevention of VAP.

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Self-expandable metallic stents for malignant biliary obstruction: Efficacy on proximal and distal tumors

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Abstract

AIM: To compare the efficacy of self-expandable metallic stents (EMS) in the treatment of distal and proximal stricture of malignant biliary tumors.

METHODS: From March 1995 to June 2004, 61 patients (40 males, 21 females) with malignant biliary obstruction who received self-expandable metallic stent implantation were reviewed retrospectively. The stents were inserted by an endoscopic or percutaneous transhepatic method. We tried to place two stents in the biliary system in T or Y configuration in cases of hilar tumors with bilateral hepatic duct obstruction. The end points of the study were stent occlusion or patient death.

RESULTS: The mean time of stent patency was 421 ± 67 d in the group of proximal stricture (group I) and 168 ± 18 d in the group of distal stricture (group II). The difference was significant in borderline between the two groups ($P = 0.0567$). The mean survival time was 574 ± 76 d in group I and 182 ± 25 d in group II. There was a significant difference between the two groups ($P = 0.0005$).

CONCLUSION: EMS implantation is a feasible, palliative method for unresectable malignant biliary obstruction. The clinical efficacy of EMS in patients with proximal hilar tumors is better than that in patients with distal tumors.

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Key words: Metallic stent; Biliary malignancy

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INTRODUCTION

Biliary stent placement is the treatment of choice for malignant biliary obstruction caused by unresectable neoplasms^[1,2]. Although self-expandable metallic stents (EMS) are much more expensive than plastic stents, EMS is claimed to be superior to plastic stents in long-term stent patency^[3]. At first, when EMS is uncovered, the tumor often invades the stent via meshes of the metallic stent, resulting in stent obstruction^[1]. To overcome the problem of tumor ingrowth in uncovered metallic stents, covered EMS have been developed in the 1990s^[1,4,5]. However, complications of covered EMS, such as cholecystitis and pancreatitis, should be noted^[1,5].

Uncovered EMS are introduced into Taiwan in the 1990s to overcome the weak points of plastic stents^[2]. In our hospital, we have begun to use uncovered EMS for the treatment of unresectable malignant biliary obstruction since 1995 and covered EMS in selective cases since 2002.

Lee *et al*^[6] found that the clinical efficacy of EMS in patients with hilar tumor is superior in those with common bile duct obstruction. Rieber and Brambs^[7] demonstrated that worse results are seen in patients with pancreatic tumors and with lymph nodes metastases of the colon and gastric cancers. We have found similar trends in our practice. Therefore, we performed this study to compare the efficacy of EMS in the treatment of distal and proximal stricture of malignant biliary tumors.

MATERIALS AND METHODS

From March 1995 to June 2004, 61 patients (40 males, 21 females) with malignant biliary obstruction who received EMS implantation were reviewed retrospectively. Neoplasms were unresectable and the diagnosis was based on pathological examination or clinical and imaging findings.

The patients received endoscopic retrograde cholangiopancreatography (ERCP) or percutaneous transhepatic cholangiography (PTC) initially. Plastic stent drainage, nasobiliary drainage or PTCD was set up when the neoplasms were confirmed to be unresectable, the patients were assigned to insertion of EMS if they agreed. Wallstent (Schneider, Switzerland) and Ultraflex diamond stent (Microvasive; Boston Scientific Corporation, MA, USA) were used in our patients. EMS were inserted either by therapeutic duodenoscopy (TJF 200, Olympus, Tokyo, Japan) or by the percutaneous transhepatic approach. We tried to place two stents in

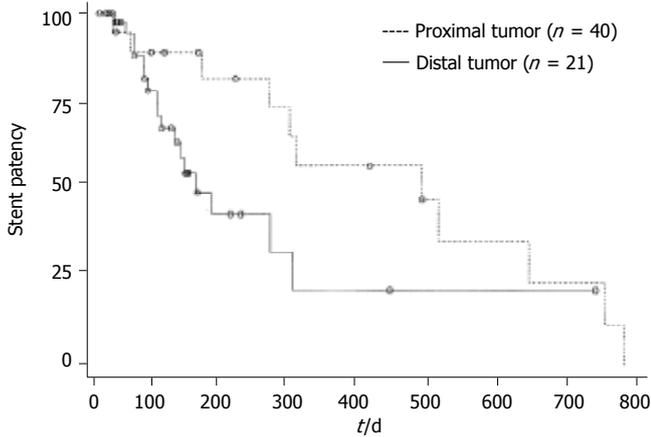


Figure 1 Kaplan-Meier graph showing cumulative stent patency. The difference was borderline significant between the two groups.

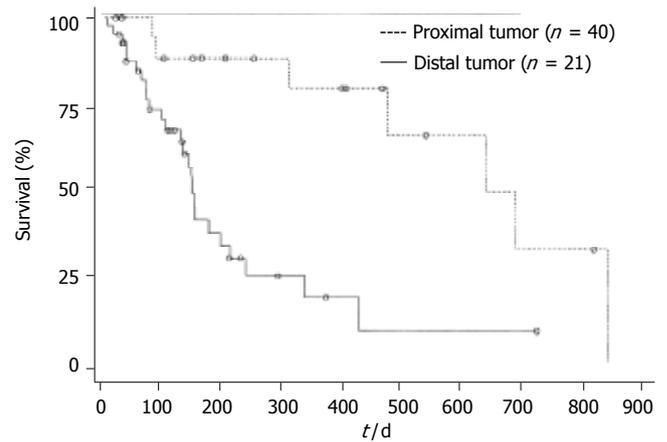


Figure 2 Kaplan-Meier graph showing survival of the patients. There was a significant difference observed between the proximal tumor and distal tumor.

the biliary system in T or Y configuration in cases of hilar tumors with bilateral hepatic duct obstruction. Covered EMS were inserted only in patients with distal stricture.

The lesions were defined as distal stricture if the tumors were located at or below the orifice of the cystic duct. The lesions were defined as proximal stricture if the tumors were located above the orifice of the cystic duct.

Stent occlusion was defined as recurrence of jaundice or cholangitis with evidence of stent stenosis requiring biliary intervention after successful insertion of EMS. The stent patency period was calculated as the time between stent placement and its occlusion or patient death. Cumulative stent patency and patient survival were evaluated by the Kaplan-Meier technique. The end points of the study were stent occlusion or patient death.

RESULTS

Patient enrollment and characteristics

Sixty-one patients were enrolled in this study. The patients were divided into two groups according to their obstruction level.

Group I (21 patients) was consisted of proximal stricture patients. The obstruction level was above the orifice of the cystic duct. The group included 19 patients with hilar cholangiocarcinoma and two patients with hepatocellular carcinoma. Twelve patients with hilar cholangiocarcinoma received two stents (in T or Y configuration) for drainage of bilateral hepatic ducts.

Group II (40 patients) was consisted of distal stricture patients. The obstruction level was at or below the orifice of the cystic duct. The group included 9 patients with cholangiocarcinoma, 17 patients with pancreatic cancers, 3 patients with ampulla of Vater cancers, 2 patients with gall bladder cancers, and 9 patients with lymph node metastases of colon cancer (2/9), gastric cancer (3/9), lung cancer (1/9), nasopharyngeal cancer (1/9), hepatocellular cancer (1/9) and laryngeal cancer (1/9).

Eight patients in group I (8/21) and 24 patients (24/40) in group II died at the time of evaluation. Covered EMS were inserted in seven patients with distal stricture and the

other 53 patients received uncovered stents.

If stent stenosis was noted during follow-up, either a second EMS (six patients), or a plastic stent through an original EMS (three patients) or PTCD (one patient) or nasobiliary drainage (three patients) was set up. However, some patients chose conservative treatment after stent occlusion.

Stent patency and survival

The mean time of stent patency was 421 ± 67 d in group I and 168 ± 18 d in group II. The difference was significant in borderline between the two groups ($P=0.0567$). The mean survival time was 574 ± 76 d in group I and 182 ± 25 d in group II. There was a significant difference between the two groups ($P=0.0005$). Cumulative stent patency and patient survival according to the Kaplan-Meier life table are shown in Figures 1 and 2.

Early complications

Early complications were defined as "complications occurring within 30 d after EMS placement". Nine cases had early complications. Seven of them belonged to distal stricture and two belonged to proximal stricture. The clinical features of early complications are listed in Table 1.

Late complications

Late complications were defined as "complications occurring after 30 d of EMS placement". A patient with hilar cholangiocarcinoma suffered from common bile duct stones 175 d after stent placement. Endoscopic sphincterotomy was performed and the stones were extracted. Gallbladder empyema was in two patients. One of them received covered EMS due to cholangiocarcinoma near the orifice of the cystic duct and symptoms occurred 66 d after stent placement. The other patient received uncovered EMS due to hilar cholangiocarcinoma and symptoms occurred 37 d after stent placement. Percutaneous transhepatic gallbladder drainage (PTGBD) relieved their symptoms. Three patients with pancreatic cancers suffered from gastric outlet obstruction (on days 80, 93 and 270 respectively) due to tumor invasion into the duodenum. Bypass surgery relieved their outlet

Table 1 Early complications after insertion of metallic stents

| Case | Complications | Type of stent | Timing of complication | Management | Result |
|------|--|----------------|------------------------|--------------------------------|-----------|
| 1 | Acute pancreatitis | Covered stent | Immediately | Conservative | Recovered |
| 2 | Acute pancreatitis | Covered stent | Immediately | Conservative | Recovered |
| 3 | Acute pancreatitis with pseudocyst | Uncoverd stent | Immediately | Percutaneous catheter drainage | Recovered |
| 4 | Inadequate expansion of stent | Uncoverd stent | 3 d | Balloon dilatation | Good |
| 5 | Inadequate expansion of stent | Uncoverd stent | 3 d | Balloon dilatation | Good |
| 6 | Acute cholangitis without Stent stenosis | Uncoverd stent | 22 d | Antibiotics | Recovered |
| 7 | Peritonitis | Uncoverd stent | 30 d | Antibiotics | Recovered |
| 8 | Stent occlusion | Uncoverd stent | 22 d | PTCD | Good |
| 9 | Subcapsular liver abscess | Uncoverd stent | 1 d | Percutaneous catheter drainage | Recovered |

Case 1-7: distal stricture.

Case 8-9: proximal stricture.

obstructions.

Complications of covered EMS

It seemed that more complications occurred in patients who received covered EMS. However, we could not arrive at any final conclusion due to the limited number of cases in our series. Acute pancreatitis occurred immediately after stent placement in two cases (2/8). Fortunately, they recovered uneventfully after conservative treatment. Stent migration (1/8) was found in a patient with an ampulla of Vater tumor 85 d after stent placement. He received conservative treatment only because of tumor infiltration in the entire second portion of the duodenum and the patient expired soon after. One patient developed gallbladder empyema (1/8) 66 d after stent placement. Her symptoms were relieved after PTGBD.

DISCUSSION

Endoscopic or percutaneous transhepatic stentplacement in the biliary tree has become a main stream in the treatment of inoperable malignant obstructive jaundice^[1]. The major drawback of plastic stents is early stent clogging and migration in spite of various modifications in the design^[2,3]. The use of EMS apparently improves the weak points of plastic stents. Although EMS is much more expensive than plastic stents, it is a cost-effective strategy^[3,8]. EMS improves patient compliance due to prolonged stent patency and less complications^[3].

According to Lee *et al.*^[6], patients with hilar obstruction have better clinical efficacy than those with common bile duct obstruction. In our study, we demonstrated similar results. Stent patency and patient survival were better in our patients with proximal stricture than in those with distal stricture. Twelve of 21 patients with proximal stricture received bilateral biliary drainage in our series. If one of the two stents were occluded, jaundice would rarely develop. However, stent occlusion would cause immediate jaundice in distal strictures.

Our study demonstrated that most of early

complications were related to the effect of stents or manipulation procedures. Acute pancreatitis(3/9) might be due to occlusion of the pancreatic duct by covered stents or secondary to the ERCP procedure. Liver abscess (1/9) might be due to the contamination of the procedure. The inadequate expansion of EMS (2/9) might be due to poor function of the metallic wires.

Most late complications were related to tumor progression. The first case with gallbladder empyema in our study might be due to the dual effects of covered stents and tumor progression. The second case with gallbladder empyema might be due to tumor progression with cystic duct occlusion. The gastric outlet obstruction in patients with pancreatic cancers was, surely due to tumor extension. Almost all stenoses of the stent and/or cholangitis are caused by tumor growth with occluded ducts, but cholangitis unrelated to stent occlusion can be noted^[11].

Although the patency of EMS is longer, there are many drawbacks after their placement, such as tumor ingrowth or overgrowth, mucosa hyperplasia induced by chronic inflammatory reaction to the stent meshes, biliary sludge and food impaction in transpapillary stents^[9]. Covered stents are significantly superior to uncovered stents by preventing tumor ingrowth^[1,4,5]. However covered stents are risky for occlusion of branch ducts (such as side branches of bile ducts, cystic ducts or pancreatic ducts), stent migration and sludge formation. Only eight of our patients with distal stricture received covered stents, and complications occurred in four of eight. Complications included acute pancreatitis (2/8), gallbladder empyema (1/8) and stent migration (1/8). A higher rate of migration is another possible disadvantage of covered stents^[10]. Due to the limited number of covered stents in our series, further studies are needed to determine the frequency of side effects in covered stents.

Because of the high cost of EMS, selection of patients and types of stents are important. Life expectancy shorter than 6 mo^[8] or tumors with liver metastases^[12] are not cost-effective for EMS placement. Although many

types of EMS are now available, which type can best improve the cost-effectiveness and quality of life remains unknown^[13-16].

In conclusion, EMS implantation is a feasible, palliative method for unresectable malignant biliary obstruction. The clinical efficacy of EMS in patients with proximal hilar tumors is superior to that in patients with distal tumors. Covered EMS is risky in regard to the complications due to pancreatitis although stent patency may be longer.

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Evaluation of contrast-enhanced computed tomographic colonography in detection of local recurrent colorectal cancer

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Abstract

AIM: To evaluate the diagnostic accuracy, sensitivity, specificity of contrast-enhanced computed tomographic colonography in detecting local recurrence of colorectal cancer.

METHODS: From January 2000 to December 2004, 434 patients after potentially curative resection for invasive colorectal cancer were followed up for a period ranging from 20 to 55 mo. Eighty of the four hundred and thirty-four patients showing strong clinical evidence for recurring colorectal cancer during the last follow-up were enrolled in this study. Each patient underwent contrast-enhanced computed tomographic colonography and colonoscopy on the same day. Any lesions, biopsies, identified during the colonoscopic examination, immediate complications and the duration of the procedure were recorded. The results of contrast-enhanced computed tomographic colonography were evaluated by comparing to those of colonoscopy, surgical finding, and clinical follow-up.

RESULTS: Contrast-enhanced computed tomographic colonography had a sensitivity of 100%, a specificity of 83% and an overall accuracy of 94% in detecting local recurrent colorectal cancer.

CONCLUSION: Conventional colonoscopy and contrast-enhanced tomographic colonography can complement each other in detecting local recurrence of colorectal cancer.

INTRODUCTION

Colorectal cancer has the third highest incidence of all cancers worldwide. Approximately 70% of colorectal cancer patients can undergo potentially curative surgical resection. Unfortunately, colorectal cancer recurs in 30% of these patients. With the advent of more aggressive surgical resection for recurrent colorectal cancer^[1], early detection of recurrent cancers while they are still limited to a local site is important to improve the patient's survival. If radical resection of locally recurrent colorectal cancer is performed before distant metastatic or unresectable disease develops, one-third to one-half of patients can increase their survival time. However, potentially curative surgery is followed by a period of uncertainty as to whether the operation has successfully cured the cancer. Treatment failure is usually apparent during the first 3 years after surgery. The precise post-operative surveillance procedures^[2] are based on clinical assessment, CEA, colonoscopy, ultrasound and computerized tomography depending on the site of primary tumor. The role of follow-up in the early diagnosis of recurrent colorectal cancer in patients having undergone resection has been investigated extensively. A large array of screening tests is available for detecting recurrent colorectal cancer, but each test has its particular limitations. Computed tomographic colonography is a new method to exploit recent developments in image acquisition which applies algorithms of virtual-reality systems to build three-dimensional models of the inner surface of the colon tube thereby simulating the conventional colonoscopic view^[3-5]. The colon wall and pericolonic structures can also be detected at the same time. Computed tomographic colonography has a high accuracy in detecting colonic neoplasia^[6-8]. Like computed tomography, contrast-

enhanced computed tomography is performed after a patient receives an air enema, and uses a narrow collimation and reconstruction interval to detect colonic lesions. Contrast-enhanced computed tomographic colonography theoretically has the ability to detect local cancer recurrence by examining both the colonic mucosa and the pericolic tissue. The use of IV contrast material in contrast-enhanced computed tomographic colonography facilitates a thorough examination of metastatic disease in solid organs. Contrast-enhanced computed tomographic colonography can display both mucosa and extramucosal local recurrence, metachronous polyps and cancers, hepatic and peritoneal metastasis^[9]. This study aimed to assess the diagnostic accuracy, sensitivity, and specificity of contrast-enhanced computed tomographic colonography in detecting local recurrence of colorectal cancer following curative resection.

MATERIALS AND METHODS

Patients

From January 2000 to December 2004, 434 patients who underwent potentially curative resection for invasive colorectal cancer (181 stage B, 253 stage C) were followed up for a period ranging from 20 to 55 mo. Eighty of the four hundred and thirty-four patients showed strong clinical evidence for recurring colorectal cancer at the last follow-up. Patients with an end or diverting colostomy or those who had contraindications for IV contrast dye were excluded.

Methods

Eighty patients who were sent for conventional colonoscopy and agreed to receive contrast-enhanced computed tomographic colonography were enrolled in this study. Written informed consent forms for both conventional colonoscopy and contrast-enhanced computed tomographic colonography were obtained from each patient. The average age of the patients was 64 years (range, 28-82 years). The ratio of male to female was 43:37. The clinical manifestations of local recurrence included bloody stools, increased serum CEA level (≥ 50 ng/mL), abdominal mass and colonic obstruction. Previous resection for invasive colorectal cancer included stages B and C of rectal cancer and colon cancer. Each patient underwent contrast-enhanced computed tomographic colonography and colonoscopy. The endoscopist was not informed of the radiological results on the same day. The average time between previous resection and contrast-enhanced computed tomographic colonography was 32 mo (range, 20-55 mo). Twenty-four hours before contrast-enhanced computed tomographic colonography, each patient received a standard bowel preparation^[10,11] consisting of 4 L of polyethylene glycol solution and 25 mg bisacodyl tablets. Prior to computed tomographic scanning, patients were placed in a left lateral decubitus position on the computed tomographic table for the introduction of a rectal enema tube. After insertion of the rectal tube, the colon was inflated with room air to patient tolerance. To reduce bowel peristalsis and colon spasms, 20 mg of buscopan was administered intravenously immediately before air

insufflation. Patient's tolerance with regard to the volume of insufflated air was measured (range, 1 500-2 000 cm³). Adequate colonic distention was checked with a computed tomographic scout. If inadequate distention developed at any colon segment, idiosyncratic positioning or additional air insufflation was performed to improve colonic distention in the collapsed regions. Contrast-enhanced computed tomographic colonography was performed employing 150 mL of Isovue-300 (Bracco Diagnostics, Princeton, NJ, USA) IV contrast medium injected at a rate of 3-5 mL/s. Images were acquired 70 s after the injection.

Computed tomographic examinations were conducted with Sightspeed Plus and Sightspeed QX/i computed tomographic scanners (General Electric Medical Systems). The patient was first examined in the supine position, and then in the prone position. Images were acquired with a 5-mm beam collimation (table speed of 10 mm/s, and reconstruction slice overlap of 2.5 mm, 230-260 mA, 120 kV). A gastrointestinal radiologist with experience in computed tomographic colonography analyzed the volumetric computed tomographic datasets using a Sun Advantage Windows (General Electric Medical Systems)^[12,13] and the General Electric Navigator program that reformats the axial two-dimensional multiplanar and three-dimensional endoluminal images^[14,15] and allowed for comparison of supine and prone datasets. Local recurrence was rated as either present, absent or indeterminate. Local recurrence was recorded as present when the characteristic appearance of an enhancing, primary extracolonic mass on intraluminal masses at or near the surgical anastomosis with or without adjacent adenopathy was identified by the contrast-enhanced computed tomography. Predominantly intraluminal abnormalities at the anastomosis were considered indeterminate for local recurrence. The liver, peritoneum, retroperitoneum, lung bases, and lymph nodes were also evaluated for the presence of metastatic disease. The colonoscopic examination was performed 2 h after contrast-enhanced computed tomographic colonography. The incidence of lesions, immediate complications, and the overall duration of the colonoscopic examination were recorded. Examination reports being indeterminate for local recurrence on contrast-enhanced computed tomographic colonography but negative colonoscopic examinations were counted as false positive examinations in statistical analysis. The sensitivity, specificity, and accuracy of contrast-enhanced computed tomographic colonography for post-operative detection of local recurrence and metastatic colorectal cancer were estimated.

RESULTS

All the 80 patients completed the contrast-enhanced computed tomographic colonography successfully. No patients had pain or complications during the procedures. Table 1 presents the findings of the contrast-enhanced computed tomographic colonography. Examination results were as follows. Local recurrence was found in 51 patients. Seventy-five of the eighty patients had adequate colonic inflation throughout the entire colon. Two of the five remaining patients had inadequate transverse colon distention and three had inadequate sigmoid colon distention though additional

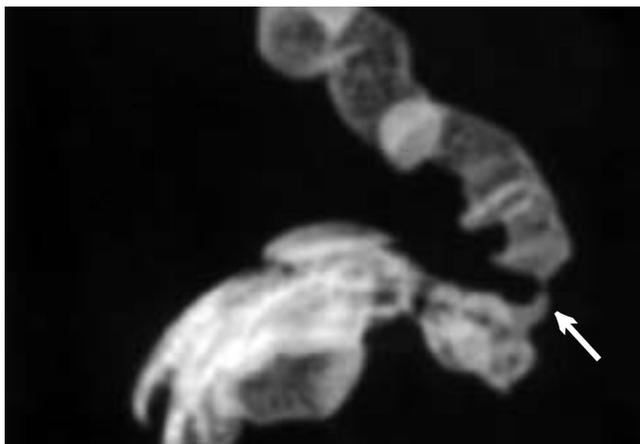


Figure 1 Virtual double contrast of the colon in a patient with local recurrence at previous anastomotic site.

air was insufflated and the positions of the patient were changed. In contrast-enhanced computed tomographic colonography, all the five patients showing thickened segmental colon wall and external luminal tumor mass compression (Figure 1) were classified as present local recurrence. The colonoscopic findings in these corresponding segmental regions showed only lumen stenosis, but no mass or mucosal lesions were found in the lumen in all the five patients. All the five patients received laparotomy for local recurrence based on a clinical presentation of three abdominal palpable masses and two colon obstructions. Surgical findings showed external colon lumen recurrent masses at previous anastomotic sites in all the five patients. Two of the five patients also had peritoneal metastasis. They all received resection of the local recurrent tumor and the colon segment with or without colostomy diversion.

Of the 51 patients with local recurrence, colonoscopic findings showed a tumor or a stricture with friable mucosa at the anastomosis, prompting a biopsy for recurrent adenocarcinoma. All the 51 patients with positive findings on both contrast-enhanced computed tomographic colonography and colonoscopy received laparotomy for local recurrence. Surgical findings showed local recurrence in all 51 patients, 35 of the 51 patients underwent segmental resection of the recurrent colorectal cancer with anastomosis, the remaining 16 patients underwent segmental resection of the colorectal cancer with colostomy diversion. All the 51 patients with local recurrence with or without liver metastasis or peritoneal metastasis received adjuvant chemotherapy after surgery.

The colonoscopic findings in the five patients which were classified as indeterminate by contrast-enhanced computed tomographic colonography revealed mucosa swelling, erythema in two patients and multiple ulcers at anastomotic site in three patients.

All the five patients underwent both contrast-enhanced computed tomographic colonography and colonoscopy 6 months later. No local recurrence or distant metastases were found, and their anastomotic sites were normal.

The colonoscopic finding in one patient, whose contrast-enhanced computed tomographic colonography

Table 1 Performance-based contrast-enhanced computed tomographic colonography findings in 80 patients

| | Patients (n) |
|---------------------|--------------|
| Local recurrence | |
| Present | 51 |
| Indeterminate | 5 |
| Not present | 24 |
| Metachronous cancer | 1 |
| Distant metastasis | |
| Liver | 8 |
| Peritoneal | 5 |

showed no local recurrence but a metachronous mass at the ascending colon, revealed a tumor at the ascending colon. The patient with metachronous cancer underwent right hemicolectomy with anastomosis. No distant metastasis or local recurrence was found in this case. The remaining 23 patients whose contrast-enhanced computed tomographic colonography did not show local recurrence were negative for colonoscopy. All the 23 patients were routinely followed up. There was no true false-negative local recurrent cancer on contrast-enhanced computed tomographic colonography. However, the five patients classified as indeterminate for local recurrence in contrast-enhanced computed tomographic colonography reports were false positive. Contrast-enhanced computed tomographic colonography had a sensitivity of 100%, a specificity of 83%, and an overall accuracy of 94% in detecting local recurrent colorectal cancer.

DISCUSSION

In patients who have undergone potentially curative colonic resections for invasive colorectal cancer, hematogenous metastases and local recurrence are the most important factors influencing prognosis. After surgery, however, there is a period of uncertainty as to whether the operation has cured the cancer or not. Treatment failure will usually be apparent during the first 2-3 years after surgery. Precise post-operative surveillance procedures, including clinical assessment, colonoscopy, abdominal computed tomography, are employed to detect recurrence of colorectal cancer. Although colonoscopy can detect intraluminal local recurrence, some local recurrences are not intraluminal and are endoscopically obscure. Abdominal computed tomography can detect hepatic and peritoneal metastases, but it is not reliable for detecting local recurrence except in those patients with a previous abdominoperineal resection. Unlike these two tests, contrast-enhanced computed tomographic colonography directly displays the anastomosis, luminal surface, colon wall and pericolonic tissues. It has, therefore, a potential to detect mucosal, intramural and extracolonic local recurrences. In our study, the overall accuracy was 94%, which is similar to that in the study by Fletcher *et al*^[16]. At the same time, it is also advantageous over the colonoscopy for detecting extracolonic local recurrence and peritoneal metastasis. In this study, 46 of the 51 local recurrences developed from the extralumen soft tissue and local lymph nodes, nearly previous anastomotic area. At the same time, 40 of the 51 cases were rectal can-

cer and 47 of stage C at their original primary cancer. It may be the reasons that were related to high local recurrent rate of our samples. As shown in our study, 5 of the 51 patients (10%) who had local recurrence detected by contrast-enhanced computed tomographic colonography had no intraluminal recurrence by colonoscopic examination.

However, all the five patients received laparotomy for local recurrence of abdominal mass and intestinal obstruction. External colon lumen local recurrence with or without peritoneal metastasis was found during surgery. Contrast-enhanced computed tomographic colonography may also show the structure of the colon when colonoscopy is incomplete^[17]. Although contrast-enhanced computed tomographic colonography has a high sensitivity (100%) for local recurrent colorectal cancer, its specificity is only 83% as shown in our study. This may be due to the inability of contrast-enhanced computed tomographic colonography to distinguish local recurrence from inflammation when enhancing soft tissue is present. Our results showed that five patients, classified as indeterminate by contrast-enhanced computed tomographic colonography, had colonoscopic findings of mucosa swelling, erythema or multiple ulcers. No local recurrence was identified in any of these five patients during the subsequent follow-up. These indeterminate conditions were then considered as false positive examinations. Another significant difference in this technique in comparison to colonoscopy is that a biopsy cannot be taken during contrast-enhanced computed tomographic colonography. However, it is recognized that contrast-enhanced computed tomographic colonography is more accurate in detecting extraluminal recurrent tumor than conventional colonoscopy.

In conclusion, contrast-enhanced computed tomographic colonography has several advantages over alternative tests in detecting local recurrent colorectal cancer. It can be a very helpful adjuvant method to colonoscopy in detecting extraluminal local recurrence, peritoneal carcinomatosis and distant metastasis. With regard to the threat of colorectal cancer and the early detection of local recurrence and distant metastasis in patients who have undergone potentially curative colonic resections for invasive colorectal cancers, conventional colonoscopy and contrast-enhanced tomographic colonography can complement each other.

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Influence of HBcAg in liver cell plasma on expression of transforming growth factor-beta 1 in liver tissue of low-grade chronic hepatitis B patients

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INTRODUCTION

The incidence rate of chronic hepatitis B (CHB) is high in China. The status of virus replication and the process of hepatic fibrosis are regarded as important. At present, TGF- β 1 is known not only as a cytokine which adjusts proliferation, development, conversion, and differentiation of cells, but also as an important transmitter of hepatic fibrosis. It plays an important role in the formation of cirrhosis^[1-3]. The synthesis and degradation of extracellular matrix (ECM) are adjusted by it^[4,5]. This study was to observe the influence of HBcAg on the expression of TGF- β 1 in liver tissue of low-grade CHB patients.

MATERIALS AND METHODS

Reagents

Mouse anti-human TGF- β 1 antibody was purchased from Fuzhou Maxim Biotechnology Co., Ltd (Lot No.: 30212238L). The PV-9000 kit was provided by Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd. Rabbit anti-human HBcAg was purchased from Fuzhou Maxim Biotechnology Co., Ltd.

Clinical data

A total of 93 low-grade CHB patients (68 males and 25 females, mean age 33.3 years, ranging from 17 to 56 years) were analyzed. HBcAg was expressed as plasma type in the liver tissue of 50 cases and no HBcAg was expressed in 43 cases. The diagnosis of all cases was coincident with the program of prevention and cure for viral hepatitis^[6].

Liver biopsy

The liver biopsy was taken from 93 cases under B ultrasound guidance. The liver tissue longer than 1.0 cm and without break was fixed by formaldehyde solution and embedded in paraffin. Six serial sections (4 μ m thick) were prepared for HE, Masson, Gordon Sweet, HBsAg, HBcAg, and TGF- β 1.

Abstract

AIM: To study the influence of HBcAg on the expression of transforming growth factor-beta 1 (TGF- β 1) in liver tissue of low-grade chronic hepatitis B (CHB) patients.

METHODS: The expression of TGF- β 1 and HBcAg in liver samples from 93 low-grade CHB patients was detected by immunohistochemistry and valuated by semi-quantitative scoring.

RESULTS: In the 93 low-grade CHB patients, HBcAg was expressed in cell plasma but not in the liver tissue. There was no significant difference between the two groups.

CONCLUSION: The expression of TGF- β 1 is not related with HBcAg expressed as plasma type in the tissues of low-grade CHB patients.

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Key words: HBcAg; Factor-beta 1; Chronic hepatitis B

Liu YG, Lu JH, Wang XX, Yang JL, Lang ZW, Meng X, Zhang LJ, Sun L, Zhang SJ, Li JQ, Song CZ. Influence of HBcAg in liver cell plasma on expression of transforming growth factor-beta 1 in liver tissue of low-grade chronic hepatitis B patients. *World J Gastroenterol*

Table 1 Influence of HBcAg in liver cell plasma on the expression of TGF- β 1 in liver tissue of low-grade CHB patients

| Groups | Expression of TGF- β 1 protein by semi-quantitative scoring(Score) | | | | | Total |
|-------------------------------------|--|---|----|----|---|-------|
| | 0 | 1 | 2 | 3 | 4 | |
| HBcAg expression in cell plasma | 2 | 2 | 19 | 23 | 4 | 50 |
| No HBcAg expression in liver tissue | 4 | 2 | 13 | 20 | 4 | 43 |

Immunohistochemistry

TGF- β 1 antigen was repaired by microwave in pH 6.0 citrate solutions. The next procedure was performed according to the instructions of PV-9000 kit. TGF- β 1 was observed randomly at least in five portal areas under 200 light microscope and the expression of TGF- β 1 was evaluated with semi-quantitative scoring method: score 0: no stain or no cell was hyperchromatic or the positive cells were less than 1% of total liver tissues; score 1-4: the areas of positive cells in hepatic lobules, hepatic sinusoid, portal areas, and fibrous plate were 1%-9%, 10%-15%, 16%-20% and more than 20% of total liver tissues, respectively^[7].

Statistical analysis

Statistical analyses were carried out with the rank test.

RESULTS

TGF- β 1 expression

The positive cells of TGF- β 1 were mainly distributed over the focal necrosis and the active fibrosis areas. They were mainly expressed in the interstitial cells of hepatic sinusoid and the inflammatory cells of portal areas. Some bile duct cells and plasma hepatocytes were also expressed.

HBcAg expression

In the 93 low-grade CHB patients, HBcAg was expressed in cell plasma but not in the liver tissue. There was no significant difference between the two groups ($H = 0.004$, $P > 0.05$, Table 1).

DISCUSSION

TGF- β 1 is a cluster of active polypeptides with closely correlative structures and similar functions. Five isomers (TGF- β 1-5) have been found though TGF- β 1 is the main content of TGF- β in human liver and has important functions. It mainly comes from the Kupffer cells (KCs) though hepatic stellate cells (HSCs) can autocrine TGF- β 1. TGF- β 1 can transfer anti-signals of cell cycle with Smad molecules and inhibit gene transcription of cell cycle correlative proteins^[8]. It can inhibit the expression of cyclin related to P70^{s6k} through P70^{s6k} (serine/threonine kinase)^[9]. The upregulation of connective tissue growth factor (CTGF) expression is related to TGF- β 1^[10] and it may be the core of activation in HSCs. TGF- β 1 can inhibit the proliferation of quiescent HSCs but cannot inhibit the activated HSCs^[11]. In CHB, its serum level is increased^[12-15]

and its expression in the liver is reinforced^[16-18]. TGF- β 1 is one of the network cytokines related to hepatic fibrosis and can accelerate the synthesis of ECM and inhibit the degradation of ECM^[19]. Following the expression of TGF- β 1, the proliferating cell nuclear antigen (PCNA) decreases in the liver^[20]. TGF- β 1 can inhibit regeneration of hepatocytes^[21] and accelerate apoptosis of hepatocytes^[22-24], but not HCC cells^[25]. Powell *et al*^[26] showed that the risk of developing cirrhosis is higher in hyper-expression than in hypo-expression of TGF- β 1.

Some scholars have found that the expression of TGF- β 1 in the liver tissues is not related with HBcAg and HBV DNA in the serum of CHB patients^[27,28]. In our study, TGF- β 1 in the liver tissue of low-grade CHB patients did not influence the status of inflammation and fibrosis with the comparability improved. We found that the expression of TGF- β 1 evaluated by semi-quantitative scoring was not related with HBcAg expression in liver cell plasma of low-grade chronic hepatitis B. We suppose that the expression of some cytokines is not related with hepatitis B virus possibly due to the role of virus replication and body immune response.

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RAPID COMMUNICATION

Clinical application of plasma shock wave lithotripsy in treating impacted stones in the bile duct system

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Abstract

AIM: To verify the safety and efficacy of plasma shock wave lithotripsy (PSWL) in fragmenting impacted stones in the bile duct system.

METHODS: From September 1988 to April 2005, 67 patients (26 men and 41 women) with impacted stones underwent various biliary operations with tube (or T-tube) drainage. Remnant and impacted stones in the bile duct system found by cholangiography after the operation were fragmented by PSWL and choledochofiberscopy. A total of 201 impacted stones were fragmented by PSWL setting the voltage at 2.5-3.5 kV, and the energy output at 2-3 J for each pulse of PSWL. Then the fragmented stones were extracted by choledochofiberscopy. The safety and efficacy of PSWL were observed during and after the procedure.

RESULTS: One hundred and ninety-nine of 201 impacted stones (99.0%) in the bile duct system were successfully fragmented using PSWL and extracted by choledochofiberscopy. The stone clearance rate for patients was 97% (65/67). Ten patients felt mild pain in the right upper quadrant of the abdomen, and could tolerate it well. Eleven patients had a small amount of bleeding from the mucosa of the bile duct. The bleeding was transient and stopped spontaneously within 2 min of normal saline irrigation. There were no significant complications during and after the procedure.

CONCLUSION: PSWL is a safe and effective method for fragmenting impacted stones in the bile duct system.

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Key words: Impacted stone; Plasma shock wave lithotripsy; Choledochofiberscopy

INTRODUCTION

Primary bile duct stones, especially intrahepatic ones, are common findings in Asian patients and challenging problems encountered in biliary surgery^[1,2]. Retained and recurrent stones represent the two main problems in the surgical treatment of stones. With the application of intra- and postoperative choledochofiberscopy, the incidence of retained stones after surgery has been markedly reduced. Though many postoperative remnant stones can be extracted via choledochofiberscopy, it remains difficult to extract impacted stones or very large stones^[3]. Impacted stones preclude insertion of the Dormia basket and cannot be captured with conventional techniques via choledochofiberscopy. Since large stones cannot pass through the T-tube fistula, it is both time consuming and frustrating to remove such stones. In order to solve this problem, we combined plasma shock wave lithotripsy (PSWL) with choledochofiberscopy. Both *in vitro* and *in vivo* studies have been conducted since the 1980s to test the safety and efficacy of this technique and the combination is widely used in clinical practice^[4].

MATERIALS AND METHODS

Patients

From September 1988 to April 2005, 67 patients (26 men and 41 women) with impacted stones or very large stones in the bile duct system underwent PSWL in combination with choledochofiberscopy in our hospital. The mean age of the patients involved was 53 ± 3 years (range, 26-83 years). Of the 67 patients, 3 had a diagnosis of acute cholecystitis with cholelithiasis and previously underwent cholecystostomy with tube drainage, 36 patients had choledocholithiasis and underwent choledocholithotomy and common bile duct exploration with T-tube drainage, and 28 patients had a diagnosis of hepatolithiasis with or without stones in the extra-hepatic bile duct and received common bile duct exploration, intrahepatic bile duct stone removal via the common bile duct with the use of stone

Table 1 Sites of impacted or huge remnant stones

| Sites | Impacted stones, <i>n</i> (%) |
|--------------------------------|-------------------------------|
| Distal end of common bile duct | 22 (10.9) |
| Common bile duct | 14 (7.0) |
| Cyst duct | 3 (1.5) |
| Hilus of hepatic duct | 10 (5.0) |
| Left hepatic duct | 24 (11.9) |
| Left internal hepatic duct | 71 (35.3) |
| Left external hepatic duct | 8 (4.0) |
| Right hepatic duct | 14 (7.0) |
| Right anterior hepatic duct | 3 (1.5) |
| Right posterior hepatic duct | 32 (15.9) |
| Total | 201 (100) |

forceps and partial hepatectomy as well as T-tube drainage when necessary. No cholangiocarcinoma was encountered in these patients. In our study, 39 patients were transferred from other hospitals. About eight attempts or so were made to extract choledochofiberscopic stones. Twenty-eight patients were admitted to our hospital at the beginning of their treatment.

Methods

All the patients underwent PSWL combined with choledochofiberscopic stone extraction without any anesthesia or sedation. The drainage tube was removed from the gallbladder or from the common bile duct, and a flexible choledochofiberscope (model CHF-T₂₀), 6 mm in diameter with a 2.6-mm working channel (Olympus, Tokyo, Japan), was inserted through the drainage fistula into the gallbladder or the bile duct system. Once the impacted or large remnant stones were found, the PSWL probe (co-designed by the Institute of Physics at the Chinese Academy of Sciences and the Department of Surgery at the Third Hospital of Peking University) was inserted into the sites of the stones through the working channel of a choledochofiberscope. The tip of the PSWL probe (length, 100 cm; diameter 2 mm, flexibility similar to that of the catheter of Dormia basket) was targeted at the impacted stones and kept approximately 5 mm away. On the basis of the build-in PSWL circuit, the voltage switch was set at 2.5–3.5 kV and the energy output at 2–3 J for each pulse of PSWL. The number of PSWL pulses sufficient to break down a stone varied in each case. The treatment was continued until the impacted stones were fragmented sufficiently by PSWL to permit extraction with the Dormia basket (Olympus, SCOP Medicine, Tokyo, Japan) or passage into the duodenum via the sphincter of Oddi with normal saline perfusion. The bile ducts were irrigated during the procedure with normal saline and gentamycin, 4 U per 500 mL of normal saline, through the working channel of a choledochofiberscope^[5].

RESULTS

In the 67 patients, 201 impacted or very large remnant stones were found in the bile ducts. The locations of stones are shown in Table 1. The gross appearance of the stones (39/201, 19.4%) found in the extrahepatic bile duct was consistent with that of the cholesterol stones. The

stones found in the intrahepatic bile duct (162/201, 80.6%) resembled the pigment stones. We measured the size of impacted stones by direct visualization on cholangiogram. No difference in stone size was found between cholesterol and pigment stone groups. The size ranged from 5 to 50 mm in diameter, with 16 stones smaller than 10 mm, 167 stones between 10 and 20 mm, 16 between 21 and 30 mm, and 2 larger than 30 mm in diameter, respectively.

In our study, 199 of the 201 stones in the extra- and intrahepatic bile ducts of 67 patients were fragmented successfully by PSWL, and then extracted under a choledochofiberscope. Each PSWL procedure took several minutes to half an hour. The success rate of fragmentation with PSWL was 99.0% (199/201). Twenty-one stones required fewer than 10 pulses of PSWL sparks for fragmentation, 65 stones 11–50 pulses, 78 stones 51–100 pulses and 37 stones more than 100 pulses. The maximum number of PSWL sparks required was 700 pulses and the minimum was only two. The average number of pulses used was 52 ± 151 .

Of the 201 stones, two were not fragmented by PSWL, one remained unfragmented though four procedures of PSWL totaling 1 063 pulses were carried out. A repeat operation was necessary for this failed PSWL. A huge impacted hard stone, 50 mm in diameter, was found in the left internal hepatic duct, which was not amenable to extraction with stone forceps. The left hepatic duct was opened and the large pigment stone was eventually extracted via a bile duct incision. Another impacted stone in the neck of the gallbladder was not fragmented by PSWL because the patient refused to fragment it by PSWL. Cholecystectomy was performed for this patient at last and the tightly impacted cholesterol stone was extracted from an incision at the neck of the gallbladder.

In 65 of the 67 patients, the fragmented stones were extracted successfully using a Dormia basket which was inserted into the bile duct through the working channel of a choledochofiberscope. The stone clearance rate was 97% (65/67). In 35 patients, only one choledochofiberscope procedure was needed to achieve clearance of remnant stones, whereas 22 patients required 2 to 5 procedures, 7 patients 6 to 10 procedures and 1 patient 18 procedures. The average number of procedures was 2 ± 41 . A total of 171 procedures were performed.

In the process of fragmenting stones with PSWL via choledochofiberscopy, all the patients felt vibration. Ten patients felt mild pain in the right upper quadrant of the abdomen and could tolerate it well. Eleven patients had a small amount of bleeding from the mucosa of the extra- and intrahepatic bile ducts. It was thought that the bleeding was induced by the pulse of PSWL sparks. The bleeding was transient and stopped spontaneously within 2 min of normal saline irrigation. No other serious PSWL-related complications occurred during and after the procedure.

DISCUSSION

Impacted stone is one of the challenging problems in biliary surgery. Before the advent of PSWL, impacted stones or very large stones were removed using biopsy forceps through the working channel of a choled-

ochofiberscope. The procedure is time-consuming and often frustrating. To solve this problem, we designed the PSWL in 1980s and have conducted a series of experiments both *in vitro* and *in vivo* to test its safety and efficacy before its application in clinical practice. Fresh cholesterol and pigment stones can be fragmented effectively both *in vitro* and *in vivo* by PSWL^[4].

Plasma shock wave lithotripsy uses magnetic pressure ($F = B^2/8\pi$) exerted on plasma. The plasma shock wave is derived from the magnetic pressure. The total magnetic energy is constant. Since magnetic field B can be increased by decreasing the area in which B exists, a stronger wave can be achieved with low energy. PSWL has three advantages. First, there is no impulse to luminal wall when PSWL is used to break down gallstones within the lumen. Second, when PSWL is combined with choledochofiberscopy, there is no heat injury and no vapor to obscure the visual field of a choledochofiberscope due to its low energy. Third, PSWL has its selection when it acts on an elastic buffer. Fragmentation of gallstones is achieved by impulsion of PSWL. When impulsion acts on elastomer, the fragmentation is selective. Therefore, PSWL can effectively break down nonelastic stones, while leaving the elastic soft tissue intact^[5].

In our study, 38 impacted or large cholesterol stones and 161 pigment stones were fragmented successfully by PSWL in the extra- and intra-hepatic bile ducts. The success rate of fragmentation was 99.0% (199/201), and the success rate of stone clearance was 97% (65/67).

Only two stones were not fragmented by PSWL. One impacted stone in the intrahepatic bile duct could not be fragmented by multiple procedures of PSWL with a total of 1 063 pulses of PSWL sparks delivered. Re-operation was performed for the involved patient, and the stone was too hard and too large (diameter, 50 mm) to be fragmented and extracted with stone forceps. The intrahepatic bile duct was opened and the stone was removed manually. Another cholesterol stone at the neck of the gallbladder was not fragmented by two procedures of PSWL with 84 pulses of PSWL sparks delivered. Cholecystectomy was performed for this patient, and a large hard cholesterol stone (diameter, 30 mm) at the neck of the gallbladder was removed.

In the present study, we successfully fragmented 199 impacted stones in extra- and intrahepatic bile ducts using PSWL when the conventional methods failed to remove them. The voltage switch was set at 2.5 - 3.5 kV, and the energy output was controlled within the range of 2 - 3 J at each pulse of PSWL. It may be very difficult to put a choledochofiberscope at the site of an impacted stone due to branches, strictures and angles of intrahepatic bile ducts. Therefore, it is unavoidable to spark directly on the impacted stone and the wall of bile duct in performing lithotripsy. In our study, 11 sites of intrahepatic bile ducts were sparked directly using the PSWL probe. Minor bleeding from the inflammatory mucosa of the bile duct occurred, and the bleeding stopped spontaneously within 2 min. No serious complications were found during and after the treatment, indicating that PSWL is a very safe method for breaking down stones *in vivo*.

The PSWL probe is flexible and can be easily placed at

the site of impacted stones to fragment the stones through the working channel of a choledochofiberscope. The position of the probe tip can be adjusted by pulling it back and forth through the working channel. The best position is 5 mm away from the stone. At this position, the PSWL probe can release energy most effectively.

Electrohydraulic shock wave lithotripsy (ESWL) and PSWL have their similarities and differences. Using discharge in fluid to induce high-amplitude hydraulic pressure waves of varying wavelengths, ESWL can fragment stones extracorporeally or intracorporeally. The extracorporeal lithotripter uses the ellipsoid reflector to reflect the shock wave into the intracorporeal site to break down the stones. The ellipsoid reflector has two focus points. One is extracorporeal, where the shock wave is emitted by discharge. The other is intracorporeal, where stones are located. Stone fragmentation and clearance rates of 76%^[6] and 92%^[7] have been achieved. Our study showed that PSWL was more effective than ESWL.

It was reported that the overall complication rate for ESWL is 13.2% - 22%^[7,8]. Bleeding and perforation are the main problems. Perhaps the power of ESWL is strong enough not only to fragment stones, but also to damage the bile duct wall. Harrison *et al*^[9] reported that to avoid grave complications, the ESWL probe should not directly contact the bile duct wall. However, PSWL may safely break down the stones without damaging the bile duct wall when the PSWL probe is in contact with the bile duct wall, suggesting that the safety of PSWL is superior to that of ESWL.

Laser has been used to fragment stones in common bile duct^[10] and intrahepatic bile duct^[11,12]. Orii *et al*^[11] reported that yttrium-aluminum laser has enough power to crush pigment stones, but its efficacy on cholesterol stones is not satisfactory. Prat *et al*^[13] reported that bile duct stones can be fragmented by laser lithotripsy. The overall success rate for stone clearance is 87.5% and the complication rate is 18.8%. Harris *et al*^[14] reported that the success rate for fragmentation of stones by laser lithotripsy is 96%, whereas the complication rate is 28%. The complications may be due to the impact of laser fiber tip on the bile duct wall^[13]. Therefore, care must be taken to advance the laser filament to the end of the scope, with the scope straight outside the patient. The relatively rigid, sharp filament may perforate the side wall of the working channel if it is advanced with force through a bent scope. Firing the laser, while the tip of the filament is inside the working channel can also damage the lining of the channel^[14]. These shortcomings limit the efficacy of laser lithotripsy. Hochberger *et al*^[10] have strongly suggested that laser can be used in the gallbladder and common bile duct, but not in intrahepatic duct. In terms of safety, PSWL is also superior to laser. The use of mechanical lithotripsy is limited to the treatment of stones in the common bile duct and in the gallbladder. It cannot be used to treat intrahepatic bile duct stones^[13]. Ultrasound lithotripsy is limited to break down stones in the gallbladder because it cannot reach the bile duct^[16].

In conclusion, PSWL is a very safe and effective method for *in vivo* fragmentation of impacted stones or large remnant stones. The PSWL combined with

choledochofiberscopy, can fragment and clear most stones when a choledochofiberscope is inserted into the bile duct system.

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RAPID COMMUNICATION

Interventional therapy for acute hemorrhage in gastrointestinal tract

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Abstract

AIM: To evaluate the diagnostic angiography and therapy for acute massive hemorrhage in gastrointestinal tract.

METHODS: Twenty-five cases of acute hemorrhage in gastrointestinal tract admitted between April 2002 and September 2004 were reviewed and analyzed by angiography and embolotherapy.

RESULTS: Fifteen patients were men and ten patients were women. The Seldinger technique and method of coaxial duct were used to get access to the bleeding region. PVA particles, gelfoam, and coils were used for embolism. All bleeding sites could be confirmed and were successfully embolized. Hemostasis was achieved in all the patients without bleeding again. The cure rate was 100%.

CONCLUSION: Interventional therapy can not only ascertain the bleeding site, but also stop the bleeding. The method is simple and the effect is certain.

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Key words: Intervention; Acute gastrointestinal bleeding; Angiography; Embolization

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INTRODUCTION

Great achievements have been made in interventional therapy in China since 1980s. The method of diagnosis and therapy for hemorrhage in gastrointestinal tract have made great progress^[1]. Transcutaneous catheter embolization is widely used in the treatment of acute massive hemorrhage in gastrointestinal tract. Selective angiography can confirm the bleeding site in gastrointestinal tract. We carried out selective or superselective embolotherapy to achieve quick hemostasis. Selective angiography is the most effective measure to detect hemorrhage^[2].

MATERIALS AND METHODS

Patients

Twenty-five patients with hemorrhage in the gastrointestinal tract admitted between April 2002 and September 2004 were treated with interventional therapy. Fifteen patients were men and ten patients were women. There were 10 cases of gastric hemorrhage, 1 case of duodenal hemorrhage, 9 cases of small intestinal hemorrhage, 3 cases of colonic hemorrhage, and 2 cases of liver disruption. The major clinical manifestation was substantive bloody stools. Some cases had hematemesis. Hemorrhagic shock occurred in five cases. All patients received treatment but hemorrhage could not be controlled. The diastolic blood pressure was lower than 40 mmHg in four patients.

Equipment and materials

Angiostar Plus-type DSA machine was purchased from Siemens Company. Catheter 4F and RADISITE® SP catheter 3 F were purchased from Cook Company. The contrast medium ultravist® 370 was obtained from Schering Company. PVA particles and coils were bought from Cook Company. All patients received celiac arteriography as well as superior and inferior mesenteric arteriography to identify the bleeding artery.

Methods

The patients underwent celiac arteriography as well as superior and inferior mesenteric arteriography. Eight patients received superselective catheterization to get access to the corresponding site of feeding artery and then embolotherapy was carried out with the corresponding materials.

RESULTS

A total of 25 patients underwent angiography for



Figure 1 A 25-year-old patient with acute life-threatening gastrointestinal bleeding. **A:** Extravasation was made from a ramification of gastrointestinal artery. **B:** Superselective angiography of the bleeding artery as an aneurysm sign of a ramification belonging to gastrointestinal artery. **C:** Control angiography after embolization with 500-710 µm PVA particles and coils demonstrating complete hemostasis.

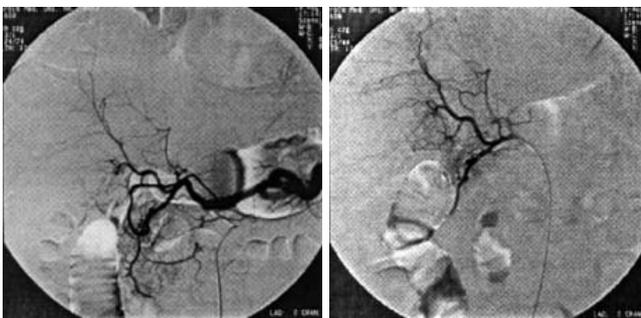


Figure 2 A 51-year-old patient with acute gastrointestinal bleeding. **A:** Contrast medium extravasation from a ramification of gastrointestinal artery. **B:** Complete hemostasis after embolization with 500-710 µm PVA particles and coils.

acute gastrointestinal bleeding. There were 15 men (60%) and 10 women (40%) (mean age 54 years, range 34-74 years). Among the 25 patients, 3 were accompanied with hematemesis, 3 had shock, 15 had bleeding from mesenteric superior artery confirmed by angiography, 2 had hemorrhage from intestinum rectum, 6 had gastric hemorrhage, and 2 had liver arteriorrhesis. Eighteen cases underwent embolism with PVA particles or PVA particles plus gelfoam and seven cases underwent coil embolism. Angiographic embolization was successful in 25 patients with gastrointestinal bleeding, and the success rate was 100%. There were no intestinal parva necrosis and other severe complications in this group (Figure 1 and 2).

DISCUSSION

Acute massive hemorrhage in gastrointestinal tract is one of the most acute abdomen^[3]. The mortality of emergency surgery is about 10%^[4]. It is difficult to identify the bleeding site and cause of hemorrhage. The treatment of hemorrhage in gastrointestinal tract includes non-operative treatment, exploratory laparotomy and interventional embolotherapy^[5,6].

Though endoscopy has been used universally, it still has some limitations in diagnosis^[7,8]. Antishock and hemostasis

can decrease hemorrhage but cannot achieve permanent hemostasis.

Intervention embolotherapy for gastrointestinal hemorrhage is a convenient and efficient microinvasive therapy^[9]. When acute hemorrhage in gastrointestinal tract occurs, hemorrhage is often massive^[10,11]. According to the leakage location of the contrast medium, we performed superselective catheterization for feeding artery embolism, which achieved hemostasis immediately, suggesting that it is a practical and effective method for old and weak patients and those who cannot tolerate operation. Embolotherapy for lower digestive tract hemorrhage is a choice of treatment^[12]. We proved that it could prevent intestinal tract ischemia.

Examination of DSA has the most important clinical value and can prevent other tissue overlapping and dynamically observe the status of artery ramification, capillaries and refluxing veins, particularly for intestinal parva as well as ascending, transverse, and descending colon. The major cause of hemorrhage is tumor and vascular malformation. Our study proved DSA could show tumor blood vessel malformation and precise image for further embolotherapy and exairesis.

Gelfoam is safer and PVA particles may be better for vascular malformation because they can achieve permanent embolism. With regard to the magnitude of PVA particles and coils, we prefer to use larger particles instead of the smaller ones.

When gastrointestinal tract hemorrhage occurs, the body constitution of patients is possibly weak and the patients usually have hemorrhagic shock^[13]. Angiography can find out the source of hemorrhage and is an important treatment modality^[14,15].

In conclusion, angiography with embolization can successfully control acute massive gastrointestinal bleeding. Embolotherapy can stop acute bleeding and prolong the life of patients.

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Effect of resveratrol on pancreatic oxygen free radicals in rats with severe acute pancreatitis

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Abstract

AIM: To investigate the therapeutic effects of resveratrol (RESV) as a free radical scavenger on experimental severe acute pancreatitis (SAP).

METHODS: Seventy-two male Sprague-Dawley rats were divided randomly into sham operation group, SAP group, and resveratrol-treated group. Pancreatitis was induced by intraductal administration of 0.1 mL/kg 4% sodium taurocholate. RESV was given intravenously at a dose of 20 mg/kg body weight. All animals were killed at 3, 6, 12 h after induction of the model. Serum amylase, pancreatic superoxide dismutase (SOD), malondialdehyde (MDA), and myeloperoxidase (MPO) were determined. Pathologic changes of the pancreas were observed under optical microscope.

RESULTS: The serum amylase, pancreatic MPO and the score of pathologic damage increased after the induction of pancreatitis, early (3, 6 h) SAP samples were characterized by decreased pancreatic SOD and increased pancreatic MDA. Resveratrol exhibited a protective effect against lipid peroxidation in cell membrane caused by oxygen free radicals in the early stage of SAP. This attenuation of the redox state impairment reduced cellular oxidative damage, as reflected by lower serum amylase, less severe pancreatic lesions, normal pancreatic MDA levels, as well as diminished neutrophil infiltration in pancreas.

CONCLUSION: RESV may exert its therapeutic effect on SAP by lowering pancreatic oxidative free radicals and reducing pancreatic tissue infiltration of neutrophils.

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Key words: Severe acute pancreatitis; Resveratrol; Oxygen free radical; Neutrophil

INTRODUCTION

Resveratrol (3,5,4'-trihydroxystilbene) is a naturally occurring phytoalexin present in grapes, fruits, and a variety of medicinal plants^[1]. It is the major active component of Rhubarb and Giant Knotweed Rhizome, etc., in traditional Chinese medicine. In *in vitro*, *ex vivo*, and *in vivo* experiments, RESV displays diverse pharmacological effects including modulation of lipoprotein metabolism and cardiovascular protection^[2], anti-inflammation^[3], platelet antiaggregatory activity^[2], antimicrobial activity^[4], antiallergic activity^[5], anticancer properties^[6,7], and most notably, antioxidant properties^[8]. In the present study, the sodium taurocholate-induced model of SAP was used to investigate the effects of RESV on SOD, MDA, MPO, serum amylase, and pancreatic pathological change to assess the role of oxidative stress in SAP and the therapeutic effects of RESV on SAP.

MATERIALS AND METHODS

Materials

RESV was obtained from Huike Botanical Development Co, stocked solution of RESV was made in Tween-80 at the concentration of 10 mg/mL and kept frozen. Sodium taurocholate was purchased from Sigma Chemical Co. SOD, MDA, and MPO assay reagents were from Nanjing Jiangcheng Bioengineering Institute.

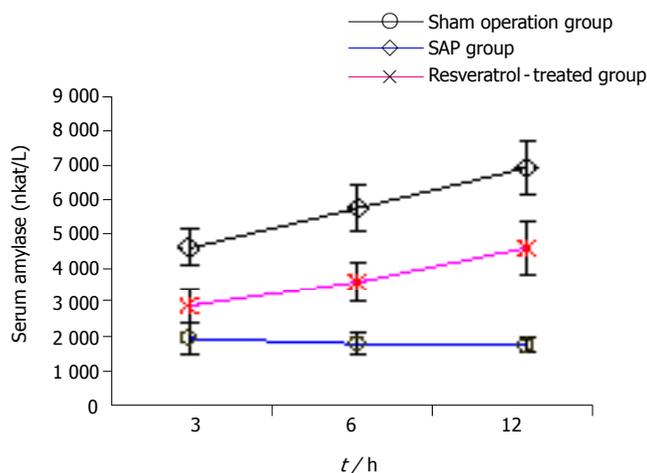
Animals

Male Sprague-Dawley rats weighing 250-300 g, purchased from Laboratory Animal Center attached to Medical College of Xi'an Jiaotong University, were used. All animals were housed in a macroion cage at 22-24 °C in a 12/12-h light/dark cycle. The animals were given a standard rat chow and fasted overnight with free access to water before the experiment. Care was provided in accordance with the "Guide for the care and use of laboratory animals" (NIH publication No. 85-23, revised in 1996). The study was approved by the Subcommittee on Experimental Animal Care of our institution.

Table 1 Pancreatic histopathologic scoring in rats (mean \pm SE)

| | <i>n</i> | Sham operation | SAP | RESV-treated |
|------|----------|-------------------|---------------------------------|--------------------------------|
| 3 h | 8 | 0.283 \pm 0.112 | 9.236 \pm 0.624 ^b | 5.283 \pm 0.646 ^d |
| 6 h | 8 | 0.219 \pm 0.171 | 11.357 \pm 0.535 ^b | 5.598 \pm 0.417 ^d |
| 12 h | 8 | 0.112 \pm 0.051 | 13.559 \pm 0.636 ^b | 6.003 \pm 0.717 ^d |

^b $P < 0.01$ vs Sham operation; ^d $P < 0.01$ vs SAP group.

**Figure 1** Comparison of serum amylase in rats.

Induction of severe acute pancreatitis

The rats were anesthetized by an intraperitoneal injection of pentobarbital (30 mg/kg, Sigma). Through a midline incision, the duodenum and the pancreatic bile duct were identified, and the duodenal wall was punctured with a 24-gauge Teflon catheter. The catheter was advanced into the common pancreatic bile duct. A microvascular clamp was placed on the duct at the hilum of the liver, a microtube clamp was placed around the catheter and the wall of the duct close to the duodenum to prevent reflux. Four percent sodium taurocholate (1 mL/kg body weight, Sigma T-0750) was injected into the pancreatic bile duct for 60 s. The clamp remained in place throughout the intraductal infusion to prevent misdirected flow into the biliary system.

Experimental design

Seventy-two rats were randomly divided into sham operation group: laparotomy followed by tipping of the pancreas without any infusion, SAP group: receiving infusion of 40 g/L sodium taurocholate into the pancreatic bile duct, RESV-treated group: perfused with RESV at a dose of 20 mg/kg body weight through vena dorsalis penis 10 min after the induction of SAP. After 3, 6, and 12 h, eight rats from each group were killed and blood was taken from the left ventricle of the heart. The samples were centrifuged (3 000 r/min, 10 min, 4°C) and serum was derivatized and immediately stored at -70°C for amylase determination. The pancreas was removed; the head of pancreas was fixed in 40 g/L paraformaldehyde for histologic analysis. Caudal pancreatic tissue was powdered using a mortar and pestle on dry ice and immediately stored

at -70°C for determining pancreatic SOD, MDA, and MPO.

Histopathologic analysis

Tissue samples of the pancreas were fixed in 40 g/L paraformaldehyde and embedded with paraffin. Five-micrometer thick sections were stained with hematoxylin/eosin and examined and graded as previously described^[9]. The total surface of the slides was scored by one blinded pathologist for four different variables (edema, acinar necrosis, hemorrhage and fat necrosis, inflammation and perivascular infiltrate) to determine the severity of pancreatic injury.

Detection of serum amylase and measurement of pancreatic SOD, MDA, and MPO

Amylase activity in serum was determined using an automatic biochemistry analyzer (Hitachi 7170). Pancreas was homogenized in physiological saline or 5 g/L HTAB using ultrasonication. The SOD content was measured using the xanthine oxidase technique based on the spectrophotometric monitoring of SOD-mediated reduction of DTNB at 550 nm. The concentration of MDA was quantified by thiobarbituric acid reaction and MPO contents were determined as described by Bhatia *et al*^[10].

Statistical analysis

Results were expressed as mean \pm SE. Statistical analysis was done using the SPSS10.0 software package. One-way analysis of variance was used to establish whether the difference among the three groups was statistically significant. $P < 0.05$ was considered statistically significant.

RESULTS

Histopathology

There was no or a small amount of clear ascitic fluid in sham operation group. More than 8 mL turbid hemorrhagic ascites could be seen in all rats of SAP group. No obvious change or slight edema could be seen in sham operation group. Pancreas in SAP group displayed disparate edema with punctiform or lamellar hemorrhage and necrosis. Saponified spots could be seen at pancreas, epiploon, mesentery, peritoneum, and perinephric fat. Pancreatic tissue was normal in sham operation group. In SAP group, pancreatic tissue displayed interstitial edema, widened lobula interspace, inflammatory cell infiltration, hemorrhage and necrosis. Microthromb could be found inside the small vessels around focal necrosis of the pancreas. In contrast, in RESV-treated group, the ascitic fluid diminished significantly and turbidity was lower than that in SAP group. Saponified spots, pancreatic edema, necrosis, inflammatory cell infiltration decreased significantly in RESV-treated group (Table 1).

Serum amylase

Compared to sham operation group, the serum amylase in SAP group increased at all time points ($P < 0.01$), decreased significantly in RESV-treated group when compared to SAP group at the corresponding time points ($P < 0.01$, Figure 1).

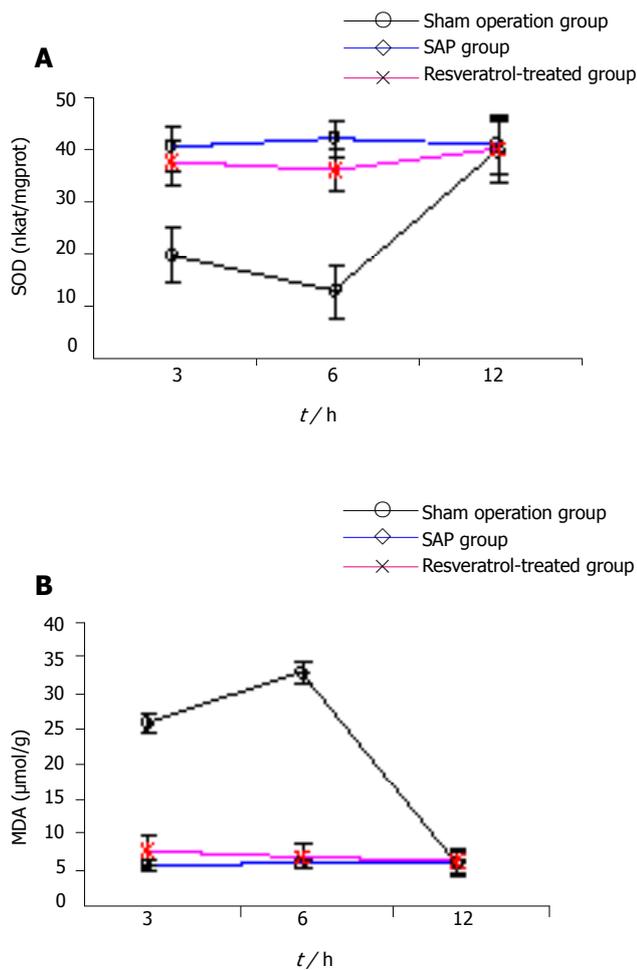


Figure 2 Comparison of pancreatic SOD(A) and MDA(B) at different time points.

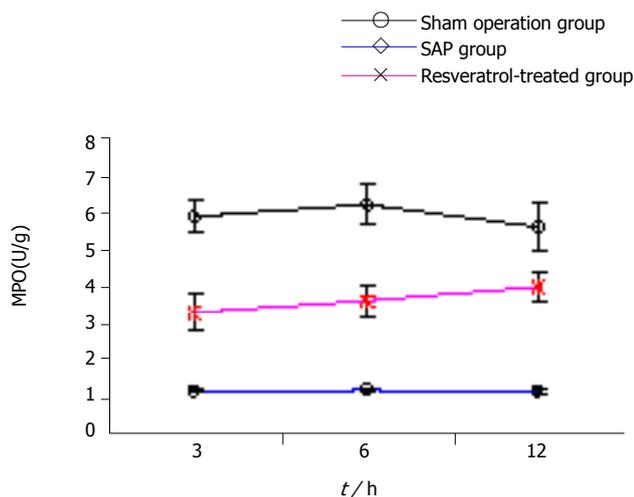


Figure 3 Comparison of pancreatic MPO at different time points.

SOD and MDA in pancreatic tissue

Compared to sham operation group, pancreatic SOD descended and MDA increased in SAP group at 3 and 6 h ($P < 0.01$), but there was no difference between the two groups at 12 h in SOD and MDA. In contrast, pancreatic SOD increased and MDA descended in RESV-treated group at 3 and 6 h when compared to SAP group ($P <$

0.01), but there was no difference in SOD and MDA at 12 h between two groups (Figures 2A and 2B).

MPO in pancreatic tissue

Compared to sham operation group, the pancreatic MPO in SAP group increased at all time points ($P < 0.01$), but decreased significantly in RESV-treated group when compared to SAP group at the corresponding time points ($P < 0.01$, Figure 3).

DISCUSSION

The pathogenesis and therapeutics of SAP are constantly emphasized in general surgery. Sanfey *et al.*^[11] have suggested a possible involvement of oxygen free radicals (OFRs) in acute pancreatitis. In 1995, Kishimoto *et al.*^[12] detected pancreatic OFRs in acute pancreatitis using the technique of chemiluminescence probe and high sensitive photon counting and found that OFRs emerge 2-3 h after the induction of acute pancreatitis, demonstrating that there exists peroxidation in acute pancreatitis. OFRs can attack polyunsaturated fatty acid's aldehyde group inside the biomembrane, initiating lipid peroxidation and accordingly forming lipid peroxidation products, as such MDA, which result in the loss of membrane stability and release of acinar cell enzyme precursors, and activate phospholipase A1 which can decompose lecithinum inside cellular membrane, further causing tissue damage. SOD is an internal antioxidant. OFRs *in vivo* are augmented when acute pancreatitis develops, which results in the consumption of antioxidant, SOD activity decrease. Therefore, it is difficult to prevent damage to the pancreas and other organs by lipid peroxidation. Detection of pancreatic SOD and MDA can reflect the peroxidation of pancreatic acinar cells and indirectly reflect the damage due to OFRs.

A number of antioxidant therapies can improve pancreatitis induced by the administration of cerulein^[13] and infusion of taurocholate^[14]. Lasztity *et al.*^[15] found that when enteral formula enriched with n-3 polyunsaturated fatty acids is used in the treatment of acute pancreatitis, the erythrocyte SOD activity is elevated significantly. Leonard *et al.*^[8] showed that RESV can scavenge OFRs as measured by spin trapping competitions using sodium formate as a second free radical scavenger, and is effective in inhibiting lipid peroxidation of cellular membranes. In the present study, when compared to sham operation group, 3 h after the induction of SAP, pancreatic SOD decreased significantly, reaching perigee at 6 h, and returned to the level of sham operation group at 12 h. In contrast, 3 h after the induction of SAP, pancreatic MDA increased significantly, reaching to apogee at 6 h, and returned to the level of sham operation group at 12 h. Simultaneously, the serum amylase and pancreatic histopathologic score increased gradually. The results indicated that overproduction of OFRs occurs in early SAP and is a significant factor for aggravating pathogenetic condition. This is coincident with the research by Reinheckel *et al.*^[16]. When compared to SAP group, pancreatic SOD in RESV-treated group increased significantly at 3 and 6

h ($P < 0.01$), whereas pancreatic MDA in RESV-treated group decreased significantly at 3 and 6 h ($P < 0.01$). On the other hand, compared to SAP group, both serum amylase and pancreatic histopathologic score in RESV-treated group decreased at all three time points ($P < 0.01$) indicating that RESV can depress earlier OFR production and lipid peroxidation of cellular membrane, diminish enzyme precursor release and necrosis of acinar cells, thus ameliorating pancreatic pathological lesions.

Neutrophils are the other major cellular source of OFRs during acute pancreatitis^[17,18], and can directly release several inflammatory cytokines, evoking systemic inflammatory reactive syndrome (SIRS). since OFRs can exert a chemoattractant effect, thereby promoting accumulation of leukocytes in the inflamed gland^[17]. Decreased acinar OFR production after RESV treatment may contribute to the reduced neutrophil infiltration, further ameliorating SIRS in SAP. In our study neutrophil sequestration within the pancreas was estimated by measuring tissue MPO activity. When compared to SAP group, the pancreatic MPO decreased at all the time points in RESV-treated group ($P < 0.01$). Moreover, studies showed that RESV can suppress the activation of NF- κ B. Thus, RESV treatment might lead to the suppression of NF- κ B activation and the subsequent prevention of several inflammatory mediator genes from being actively expressed^[19-21]. This mechanism may also help to reduce the sequestration of neutrophils in the pancreas and the associated OFR generation, thus effectively attenuating pancreatic damage.

In conclusion, overproduction of OFRs takes place in early SAP, and is a significant factor for aggravating pathogenetic condition. RESV can ameliorate pathological lesions in the pancreas by lowering pancreatic OFRs and reducing pancreatic tissue infiltration of neutrophils. It may have certain therapeutical effects on acute pancreatitis.

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Known and probable risk factors for hepatitis C infection: A case series in north-eastern Poland

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INTRODUCTION

Hepatitis C virus (HCV) is the most common chronic blood-borne infection in developed countries and the major cause of chronic liver disease, cirrhosis and hepatocellular cancer. Since no effective vaccine against HCV infection is available, reducing the spread of the disease relies on primary prevention activities that can cut the transmission routes and reduce or eliminate the risk of acquiring infection. Since its discovery in 1989, much has been learnt about the ways in which HCV is transmitted. Well-known and common modes of transmission involve transfusions received before the routine screening of blood donors was implemented (in Poland since July 1992), intravenous drug use (IVDU), hemodialysis, and occupational exposure to the infected blood in health-care facilities^[1-3]. Sexual transmission of HCV has also been demonstrated, but it is known to occur with less frequency compared to hepatitis B or HIV. Other risk factors are considered, but their role has not been established convincingly. Some case-control studies linked HCV infection to surgical or dental procedures, endoscopies, tattooing, body piercing, acupuncture, household contact with an anti-HCV person, and intranasal cocaine use. The results of the studies are, however, conflicting and some expert groups have found no associations between those exposures and HCV infections. In fact, there may be geographical differences in predominance of certain routes of transmission over others. Although the data on HCV epidemiology in Eastern Europe are scarce, the available literature and experts' opinions indicate that surgical and parenteral procedures (independent from blood transfusions) account for 40%-71% of HCV infections^[4].

The precise data on HCV prevalence in the general population in Poland is lacking since no population-based study was carried out. According to WHO estimates about 1.4% of general Polish population may be infected with HCV, which means about 560 000 persons in the whole country^[5]. At the same time, statistics of the National Institute of Hygiene in Warsaw registered about 13 000 infections in the years 1997-2003 (compulsory registration of HCV started in 1997)^[6]. It is clear that most people with HCV infection in Poland are unaware of their status, even if we assume under-reporting by medical and laboratory services.

Abstract

AIM: To describe the risk profile of patients in hospital with hepatitis C virus (HCV) infection in Poland.

METHOD: Using a structured questionnaire, all patients with confirmed HCV infection were interviewed about the risk factors.

RESULTS: Among the 250 patients studied, transfusion before 1993 was the primary risk factor in 26%, intravenous drug use setting in 9% and occupational exposure in health-care in 9%. Women were more likely to have a history of occupational exposure or transfusion before 1993 and less likely to undergo minor surgery. Known nosocomial risk factors (transfusion before 1993, dialysis) were responsible for 27% of infections, probable nosocomial factors (transfusions after 1992, minor surgery) for 14% and further 9% were occupationally acquired infections.

CONCLUSION: A careful history investigation can identify a known or probable risk factor for HCV acquisition in 59% of patients with HCV infection. Preventive activities in Poland should focus on infection control measures in health-care setting.

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Key words: Hepatitis C; Risk factors

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In Poland, in contrast to USA or UK, where drug use prevails, many HCV cases are presumed to be nosocomial infections. Previous studies based on the samples of patients hospitalized for acute or chronic hepatitis C linked as many as 59%-71% of HCV infections to medical procedures^[7]. Our recent case-controlled study aiming at identifying medical procedures associated with exposure to HCV found that transfusions (OR = 3.7, 95%CI = 2.2-6.3), minor surgery (OR = 3.2, 95%CI = 1.5-6.7) and dental care (OR = 2.3, 95%CI = 1.4-4.0) were independently associated with HCV infection^[8]. In Poland, hepatitis B virus (HBV) infection, which spreads in a similar way to HCV, is also frequently a medically linked disease^[9].

Identifying risk factors is important in order to plan preventive activities and is also necessary to target screening for people with higher pre-screening probability of the disease.

We undertook this study in order to describe the risk profile in a population of patients seeking care in a tertiary care level hospital in a defined region of Poland.

MATERIALS AND METHODS

Patients

The study took place in the Department of Infectious Diseases, Medical University of Białystok (north-eastern Poland) between June 1, 1998 and December 31, 2004. All consecutive adult patients with acute or chronic hepatitis C admitted to the department were invited to participate. This department is the biggest hepatologic center in Podlaskie Region (1 200 000 inhabitants, north-eastern Poland), where the majority of patients with chronic viral hepatitis from the whole region are referred for evaluation and antiviral treatment.

The diagnosis of hepatitis C was based on the presence of anti-HCV antibodies (ELISA, third generation test, IMx MEIA, Abbott, Chicago, USA) and was confirmed by means of HCV-RNA testing (qualitative nested RT-PCR). The standard procedures with a suspected case of chronic hepatitis C include initial testing for anti-HCV and determination of ALT levels, and repeating the tests for anti-HCV and ALT levels after 6 mo. If anti-HCV is repeatedly positive and ALT levels remain elevated above the normal range, patients are tested for HCV-RNA, and liver biopsy is performed. Since confirmatory tests using immunoblotting were not available, only the patients who were positive for anti-HCV and HCV-RNA were included in the present study.

Methods

All patients were interviewed extensively by one of the two doctors with the use of a structured questionnaire. The questionnaire covered demographic data (age, sex, education, job, place of living) and information about the possible risk factors. The risky exposures considered in our study were as follows: (1) known risk factors, such as IVDU, transfusions of blood or blood products before 1993, employment as a health-care worker with exposure to blood or other fluids, hemodialysis, and sexual contact with an anti-HCV positive person; (2) probable risk factors, such as household (non-sexual) contact with an

Table 1 Prevalence of all known and probable risk factors among 250 chronic hepatitis C patients (n, %)

| Risk factors | All n = 250 (100%) n (%) | F n = 92 (36.8%) n (%) | M n = 158 (63.2%) n (%) |
|-------------------------------------|--------------------------------|------------------------------|-------------------------------|
| Known risk factors | | | |
| IVDU | 22 (8.8) | 4 (4.4) | 18 (11.4) |
| Transfusion <1993 | 67 (26.8) | 31 (33.7) | 36 (22.8) |
| Hemodialysis | 5 (2.0) | 3 (3.3) | 2 (1.3) |
| Occupational exposure - health-care | 34 (13.6) | 21 (22.8) ¹ | 13 (8.2) ¹ |
| Sexual exposure to HCV | 2 (0.8) | 0 (0.0) | 2 (1.3) |
| Probable risk factors | | | |
| Transfusions after 1992 | 17 (6.8) | 6 (6.5) | 11 (7.0) |
| Minor surgery | 36 (14.4) | 5 (5.4) ² | 31 (19.6) ² |

¹P<0.05, F vs M in occupational exposure - health-care group; ²P<0.05, F vs M in minor surgery group.

anti-HCV positive person, transfusion after 1992, and minor surgery; and (3) other potential risk factors for HCV infection, such as surgeries, endoscopies, tattoos, previous hospitalizations, and acupuncture. In further analysis, the patients with more than one risk factor were classified as having only the risk factor according to the hierarchy. The hierarchy of risk factors used in our study was based on the data from medical literature as well as on the results of our previous study indicating the link between history of minor surgery, transfusion after 1992 and increased risk for HCV infection in Poland. We did not include dental care (which had also been associated with HCV infection) into probable risk factors because of the low specificity of that exposure with nearly 90% of the study group providing history of dental treatment. For comparisons between groups, the patients were stratified by age (<45 or ≥ 45 years) and by gender.

Statistical analysis

The statistical calculations were performed with the use of statistical package, Statistica Pl. Fisher's exact test was used for the analysis of differences in risk factors between groups (males vs females, younger vs older and younger vs older patients of the same sex). A P value < 0.05 was considered statistically significant.

RESULTS

A total of 420 anti-HCV positive individuals were evaluated during the study period and 250 were eligible for the study. In the remaining 170 cases, the results of HCV-RNA testing were either negative or unavailable. Among the study group, there were 92 females (36.8%) and 158 males (63.2%). Patients' age ranged from 18 to 70 years with the mean age of 39.7 (± 2.8) years. Females were found to be obviously older compared to males (mean age, 43.4 vs 38.4 years, P < 0.05). Majority of the patients came from urban setting (219 patients, 87.6%) and had secondary (120, 48.0%) or elementary (80, 32.0%) education.

Table 1 presents the overall prevalence of the considered

Table 2 Distribution of known and probable primary risk factors (one per person according to the hierarchy) stratified by age and gender

| Risk factors | All n (%) 250 (100%) | F n (%) 92 (36.8) | M n (%) 158 (63.2) | F < 45 n (%) 43 (46.7) | M < 45 n (%) 109 (69.0) | F > 44 n (%) 49 (53.3) | M > 44 n (%) 49 (31.0) |
|-------------------------------------|----------------------------|-------------------------|--------------------------|------------------------------|-------------------------------|------------------------------|------------------------------|
| Known risk factors | 112 (44.8) | 49 (53.3) ^a | 63 (39.9) ^a | 26 (60.5) ^c | 43 (39.4) ^c | 23 (46.9) | 20 (40.8) |
| IVDU | 22 (8.8) | 4 (4.4) | 18 (11.4) | 3 (7.0) ^e | 18 (16.5) ^e | 1 (2.0) ^e | 0 (0.0) ^e |
| Transfusion before 1993 | 65 (26.0) | 31 (33.7) ^g | 34 (21.5) ^g | 18 (41.9) ⁱ | 19 (17.4) ⁱ | 13 (26.5) | 15 (30.6) |
| Occupational exposure - health-care | 22 (8.8) | 13 (14.1) ^k | 9 (5.7) ^k | 5 (11.6) | 6 (5.5) | 8 (16.3) | 3 (6.1) |
| Dialysis | 3 (1.2) | 1 (1.1) | 2 (1.3) | 0 (0.0) | 0 (0.0) | 1 (0.0) | 2 (4.1) |
| Sexual contact with HCV | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Probable risk factors | 35 (14.0) | 6 (6.5) ^m | 29 (18.4) ^m | 4 (9.3) | 17 (15.6) | 2 (4.1) ^o | 12 (24.5) ^o |
| Transfusion after 1992 | 13 (5.2) | 4 (4.4) | 9 (5.7) | 2 (4.7) | 3 (2.8) | 2 (4.1) | 6 (12.2) |
| Minor surgery | 22 (8.8) | 2 (2.2) ^a | 20 (12.7) ^a | 2 (4.7) | 14 (12.8) | 0 (0.0) ^s | 6 (12.2) ^s |
| No known or probable risk factors | 103 (41.2) | 37 (40.2) | 66 (41.8) | 13 (30.2) | 49 (45.0) | 24 (49.0) | 17 (34.7) |

^a*P* < 0.05, known risk factors F vs M; ^c*P* < 0.05, known risk factors F < 45 vs M < 45; ^e*P* < 0.05, IVDU all < 45 vs all > 44; ^g*P* < 0.05, transfusions before 1993 F vs M; ⁱ*P* < 0.05, transfusions before 1993 F < 45 vs M < 45; ^k*P* < 0.05, occupational health-care F vs M; ^m*P* < 0.05, probable risk factors F vs M; ^o*P* < 0.05, probable risk factors F > 44 vs M > 44; ^s*P* < 0.05, minor surgery F vs M; ^t*P* < 0.05, minor surgery F > 44 vs M > 44

Table 3 Potential sources of exposure to HCV among 103 persons without known or probable risk factors

| Risk factors | All n (%) 103 (100%) | F n (%) 37 (35.9) | M n (%) 66 (64.1) |
|--------------------------------|----------------------------|-------------------------|-------------------------|
| Surgery (other than minor) | 49 (47.6) | 24 (64.9) ^a | 25 (37.9) ^a |
| Endoscopies | 52 (50.5) | 22 (59.5) | 30 (45.5) |
| Hospitalizations (more than 5) | 22 (21.4) | 15 (40.5) ^c | 7 (10.6) |
| Tattoo | 11 (10.7) | 0 (0.0) | 11 (16.7) |
| Dental care | 91 (88.3) | 33 (89.2) | 58 (87.9) |
| None of above | 1 (1.0) | 0 (0.0) | 1 (1.5) |

^a*P* < 0.05, surgery (other than minor) F vs M; ^c*P* < 0.05, hospitalizations (more than 5) F vs M; ^e*P* < 0.05, tattoo F vs M.

known and probable risk factors. In the studied hepatitis C group, there were no cases of household (non-sexual) exposures to HCV and that factor is not presented in the table. The most prevalent reason was transfusion before 1993 in 67 (26.8%) cases, followed by minor surgeries in 36 (14.4%) cases, occupational exposure in 34 (13.6%) cases during health-care and IVUDU in 22 (8.8%) cases. Table 2 displays hierarchical distribution of primary risk factors for HCV infection (each case is represented only once). Overall known risk factors could be identified in nearly 45% of patients and probable in further 14%. About 41.0% had no known or probable risk factors according to the criteria established in our study. Three factors, such as history of transfusion before 1993, occupational exposure and minor surgery, were significantly associated with gender. The first two factors occurred more frequently in females while minor surgery was more common among

males. There was a clear tendency towards more frequent occurrence of IVUDU among males, but the difference did not reach statistical significance.

Overall females were more probable than males to have known risk factors (53.3% vs 39.9%, *P* < 0.05). The prevalence of drug use in the younger group (21/152, 13.8%) was significantly higher than that in the older group (1/98, 1.0%, *P* < 0.05). IVUDU in our study was almost limited to males under the age of 45 with 82% (18/22) of patients.

In our study, females ≥45 years were the group with the highest frequency (49%) of unidentified (without known or probable risk factors) source of HCV infection, but the difference was not statistically significant.

Table 3 depicts the overall prevalence of other potential exposures to HCV among 103 patients without known or probable risk factors. Only one person denied all of the considered risk factors.

Known nosocomial risk factors (hemodialysis, transfusion before 1993) were responsible for 27% of all infections, while probable nosocomial risk factors (transfusion after 1992, minor surgery) were responsible for 14% of infections. Further 9% were occupationally acquired infections in health-care workers. Altogether at least 50% of all HCV infections in our study were associated with a health-care sector.

DISCUSSION

Hepatitis C infection affects approximately 560 000 people in Poland. Difficulties in identifying risky exposures result from the fact that most cases are clinically silent and remain undiagnosed for many years. In addition, some potential risk factors (e.g., dental care, hospitalizations) are very common and their non-specific nature hinders

establishing their role in HCV transmission and makes targeting prevention measures difficult.

The demographic characteristics of our sample were comparable to the data collected by National Institute of Hygiene in Warsaw for all 2 255 new hepatitis C cases registered in Poland in 2003 (incidence 5.90/100 000 inhabitants)^[6]. In our group, males constituted 63% of cases and males under the age of 45 made up nearly 44% of all HCV infections. The national statistics for 2003 indicate that 57% of HCV infections occurred in males, and younger males between 20 and 24 years of age had the highest incidence of infection (10.8/100 000 inhabitants). Similarly, as in our findings, majority of the registered new cases came from urban setting (80.0%). Currently, there are no national reports on the distribution of risk factors among registered HCV cases in Poland.

In our study, known risk factors could be identified in nearly 45% of HCV infections. As expected, transfusions before 1993 represented the most prevalent known exposure. Transfusion before 1993 could be documented in more than 60% of HCV infected females under the age of 45. In a majority of cases, the infection was the iatrogenic effect of postpartum iron-deficiency anemia treatment. Blood transfusion was frequently used to raise the hemoglobin levels and to allow earlier discharge from hospital.

Primary prevention activities have been already undertaken and current procedures have virtually eliminated the risk of HCV infection from blood transfusion. The knowledge of history of transfusion is important for secondary prevention, which means target screening for HCV infection. Testing should be routinely offered to the persons with the history of transfusions before 1993, accompanied by appropriate counseling and medical management.

IVDU was responsible for 9% of infections and was almost exclusively limited to males under the age of 45. At the same time, younger males made the group with high frequency of no known or probable risk factors. It may be speculated that, at least in some of those cases, incidental drug use could be responsible. Many of American blood donors found to be positive for HCV infection revealed the history of drug use, despite initial denial of such exposure^[10]. In our study, the rate of HCV infections resulting from drug use among males <45 years of age (17%) was still much lower than that reported in similar groups in the United States (59%-60%)^[11,12]. This finding confirms the primary role of nosocomial HCV spread in Poland and may reflect lower numbers of drug users in Poland compared to USA or other Western European countries. Estimates of the prevalence of problem drug use (defined as injecting drug use or long duration, regular use of opiates, cocaine and/or amphetamines) range in European Union countries between 2 and 10 cases per 1 000 of the population aged between 15 and 64 years, and Poland remains in the low range^[13]. As in many Central and Eastern European countries, the major problem regarding drug use in Poland concerns heroin. Opiate users represent the biggest proportion of persons admitted to residential treatment due to drug addiction.

Shared usage of contaminated needles and syringes results in high prevalence of HCV antibodies. Among 100 parenteral drug users in Warsaw, 76% were found to be seropositive for anti-HCV^[14]. Another possible explanation for relatively low percentage of intravenous drug users in our study might be failing secondary prevention of HCV infection among drug-dependant population in Poland. In this respect, it is possible that drug users are under-represented in the hospital samples because of the hindered access to screening procedures, health-care services and long-term antiviral treatment.

Occupational infections constituted nearly 10% of primary risk factors in our study and occurred mostly in women in both age groups. Nurses are the predominant occupational group injured by needles and other sharp-edged instruments, because they are the largest segment of the workforce in health-care, and also because they may have a higher rate of injury. This group sustains about 50% of all needle-stick injuries. The accidents typically happen when workers are recapping needles, transferring body fluids from one container to another, or when they do not dispose the used needles properly^[15,16]. In 2001, hepatitis C constituted the major cause of all occupational blood-borne infections in health-care workers in Poland^[17].

In this study, known nosocomial and probable nosocomial risk factors were responsible for 27% and 14% of all infections, respectively. Fortunately, the major nosocomial risk factor, transfusion of contaminated blood, has been virtually eliminated. After the introduction of screening of all blood donations for HCV-RNA, the calculated risk of HCV infection resulting from the transfusion of blood during window period is about 1/1 000 000 blood units^[18].

In 102 out of 103 HCV-infected individuals without known or probable risk factors, other potential exposures could be found and most of those were medically linked.

Our study confirms that hospital setting remains as an important source of infection. This seems to be a common feature in Eastern European countries. Nosocomial transmission of HCV is possible if infection-control techniques or disinfection procedures are inadequate and contaminated equipment is shared among the patients. Diagnostic or treatment procedures (surgical or parenteral procedures without blood transfusions) in hospitals were indicated as the source of infection in approximately 59%-65% cases in Poland, 59% in Latvia and 46% in Hungary^[4].

Our study had certain limitations as follows: the patients with IVDU or hemodialysis were under-represented in the sample; the percentage of patients with probable risk factors was related to our definition of those factors; and we did not include certain medical procedures or events into that category because previous case-control study did not confirm their relation to HCV infection and because some of them could not be the cause but the result of HCV infection (endoscopies, hospitalizations). In our previous study, we found an increased risk associated with minor surgery^[8]. Including that exposure into probable risk factors category could overestimate the percentage of patients in our study with known or probable risk factors. The possible overestimation was almost limited to men.

In our experience, a careful history can elicit a risk factor in nearly 60% of hepatitis C infections. The study reveals that overall of at least 50% of HCV infections are associated with health-care sector (transfusions, occupational exposure, other nosocomial exposures). Among the remaining 40% of patients without known or probable risk factors, almost all individuals provided a history of contact with health-care sector preceding diagnosis of hepatitis C. There is a clear tendency towards association of risk factors with age and gender. IVDU occurs mainly in younger males, whereas transfusion before 1993 was more common in younger females. Females are more probable to acquire the infection performing occupational activities in health-care. The two groups with the highest rates of risk factors were women > 44 years and men < 45 years. We speculate that hospitalization and surgery are responsible for some of those infections in older females and IVDU in the younger males.

We conclude that preventive activities against HCV spread in Poland should focus on infection control measures in health-care setting.

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CASE REPORT

Sigmoidorectal intussusception of adenoma of sigmoid colon treated by laparoscopic anterior resection after sponge-on-the-stick-assisted manual reduction

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after sponge-on-the-stick-assisted manual reduction. *World J Gastroenterol* 2006; 12(1): 146-149

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INTRODUCTION

Adenomatous polyp of the colon is quite a common disease, but its presentation as intussusception is very rare. The diagnosis of the underlying lesion in adult intussusception is often difficult, and it is commonly established only after surgical exploration. However, a modern imaging technique, CT, can be helpful in the precise preoperative identification of the etiology of the intussusception^[1,2]. Although surgical resection is generally indicated in most adult intussusceptions, it is still controversial whether or not the sigmoidorectal intussusception should be reduced before resection. We present here a rare case of large villotubular adenoma of the sigmoid colon as a cause of sigmoidorectal intussusception, and it was treated by laparoscopic anterior resection after a manual reduction using our own technique.

CASE REPORT

A 56-year-old male was referred by his general practitioner to our Coloproctology Clinic for further evaluation and management of an intraluminal rectal mass with intermittent rectal bleeding for one day. The sodium phosphate enema (Fleet® Enema; CB Fleet Co, Lynchburg, VA, USA) was used for bowel preparation for sigmoidoscopy in the local clinic. The local practitioner finished the sigmoidoscopy without any complications and gave a presumptive diagnosis of rectal mass with partial downward displacement of involved bowel. The rectal mass was detected at 15 cm from anal verge upon flexible sigmoidoscopy (Figure 1A), and the local practitioner did not explore the entire colon beyond the lesion. Several hours after his admission, he suddenly had lower abdominal cramping pain with rectal bleeding during bowel preparation using polyethylene glycol electrolyte solution. A digital rectal examination palpated a protuberant mass-like lesion with a smooth surface. An emergency colonoscopy revealed a sausage-like protrusion at the rectum about 3 cm proximal to the anal verge (Figure 1B),

Abstract

We present herein a case report of sigmoidorectal intussusception as an unusual case of sigmoid adenomatous polyp. The patient was a 56-year-old man who suffered from rectal bleeding for one day. He initially visited his general practitioner and was diagnosed as having an intraluminal mass of 15 cm from the anal verge. Several hours after admission to our coloproctology clinic, he suddenly presented with lower abdominal cramping pain with rectal bleeding during his bowel preparation using polyethylene glycol electrolyte solution. An emergency colonoscopy revealed that the invaginated colon with polypoid mass was protruded to the lower rectum. Gastrograffin enema showed that the invaginated bowel segment was 3 cm from the anal verge. CT scan showed the typical finding of intussusception. We performed laparoscopic anterior resection and anastomosis after the sponge-on-the-stick-assisted manual reduction. The permanent pathologic finding showed villotubular adenoma of the sigmoid colon.

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Key words: Sigmoidorectal intussusception; Adenomatous polyp; Laparoscopic resection

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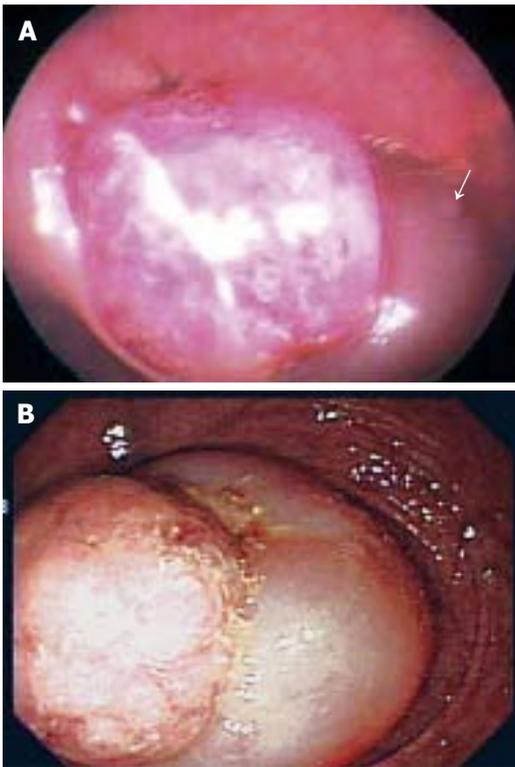


Figure 1 Endoscopic findings. **A:** Flexible sigmoidoscopy at a local clinic showing the mass lesion 15 cm from the anal verge, and the partial downward displacement of involved bowel (arrow); **B:** Colonoscopy at our clinic showing the invaginated bowel with a round mass lesion about 3 cm from the anal verge.

and our initial endoscopic diagnosis was nongangrenous sigmoidorectal intussusception that was caused by a polyp. A CT scan of the pelvis was performed to obtain a detailed delineation of the lesion. The CT showed a round target-shaped mass lesion consistent with intussusception, and the leading lesion was thought to be a benign mass with a low likelihood of malignancy (Figure 2). Our clinical impression was sigmoidorectal intussusception secondary to benign adenomatous polyp of the sigmoid colon. For the next step, we tried a contrast study using gastrograffin for the reduction as well as for the diagnostic imaging of the intussusception. This also showed an invaginated bowel segment at the lower rectum about 3 cm proximal to the anal verge (Figure 3), and a hydrostatic pressure was given via the rectum for reduction, but it was in vain.

After the general anesthesia, he was placed supine in the modified lithotomy position using Dan Allen stirrups. After the insertion of the umbilical port for the establishment of pneumoperitoneum, surgery was begun in the Trendelenburg position. On laparoscopic exploration, the neck or commencement of the intussusception was located at the rectosigmoid junction, but the distal end was not identified in the peritoneal cavity. The rectal wall of the involved segment was mildly edematous, but it was otherwise healthy on gross examination. No other intra-abdominal pathologic findings were detected. For the manual reduction of the intussusception, we tried to push up the distal end of invaginated segment with a lubricated sponge-on-the-stick from the anus while monitoring by a laparoscope. This maneuver made it



Figure 2 Contrast enhanced CT scan of pelvis showing a homogeneously well enhancing mass at the intussuscepted bowel tip.

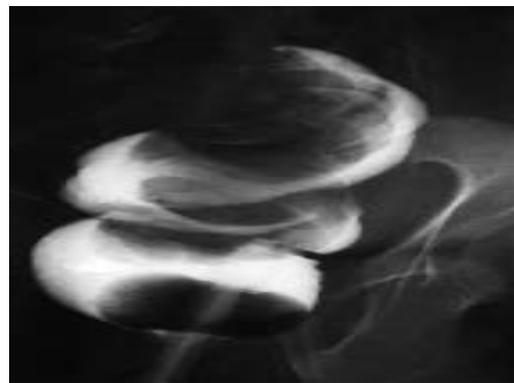


Figure 3 Gastrograffin study showing a large smooth-invaginated mass intussuscepting into the rectum.

possible to laparoscopically visualize the distal end of the intussusception in the pelvic cavity. We then pulled the intussuscepted segment out proximally using a laparoscopic bowel grasper, but this trial failed. We next tried manual reduction again by grasping the upper ring fold of the intussusception steadily by the index and middle fingers of the surgeon through a suprapubic vertical midline incision 4 cm in length while simultaneously pushing the lowest segment up using a lubricated sponge-on-the-stick via the anus; in this fashion we were successful in reducing the intussuscepted segment. The leading point of the intussusception was located in the mid-sigmoid colon. We performed laparoscopic anterior resection on an oncologic basis because the pathology of the mass had not been confirmed as being benign. After the resection of the segment, the specimen was extracted via the previous suprapubic vertical midline incision. The bowel continuity was safely restored using a standard double-stapling technique between the descending colon and midrectum. The final pathologic diagnosis was villotubular adenoma of the sigmoid colon measuring 2.5 cm × 2 cm in size (Figure 4). The patient was discharged from the hospital 8 d after the operation without any complications.

DISCUSSION

Intussusception is a quite different entity in adults from

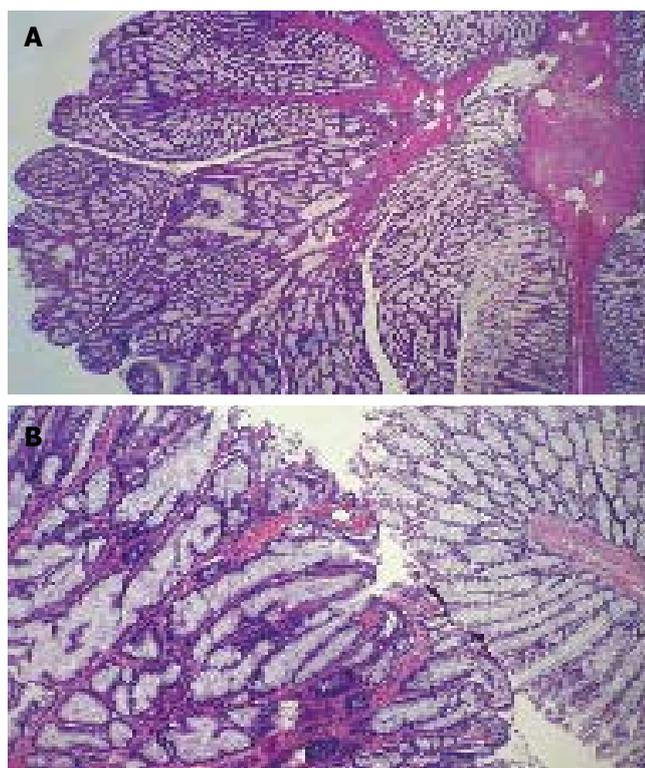


Figure 4 Hematoxylin and eosin staining of the lesions. **A:** The polypoid mass of the colon showing branching papillary projections composed of proliferated glands and thin fibrous stalks ($\times 10$); **B:** The proliferated glands of the polyp (left) lined by single or pseudostratified hyperchromatic columnar cells as compared with normal colonic mucosa (right), but without any malignant changes ($\times 40$).

that found in children. An organic etiology is found in 70%-90% of adult intussusceptions, and primary or secondary malignant neoplasia are documented in about 20%-50% of adult intussusceptions^[3-5]. In particular, intussusception occurring in large bowel is more likely to have a malignant etiology^[4]. Large polypoid adenomas and lipomas are the most common benign leading points found in adult colocolic intussusception^[5,6]. Most reported cases of sigmoido-recto-anal intussusception in the literature are caused by adenoma or carcinoma^[7-10], as compared to our case where the lowest intussuscepted segment was located in the lower rectum without protrusion through the anus.

In pathogenesis of intussusception, peristalsis and ingested food push the lesion with the adjacent bowel, which telescopes into the relaxed intestinal segment distal to it^[3]. We initially planned to perform colonoscopy after bowel preparation using the polyethylene glycol electrolyte solution, not only to inspect the entire colon but to make a more specific diagnosis of the intussusception. But based on the findings that the patient suddenly complained of lower abdominal pain during bowel preparation and that the intussusceptum was detected 3 cm apart from anal verge at our clinic contrary to the sigmoidoscopic finding that the mass was reported to be 15 cm proximal to the anal verge at previous clinic, this bowel preparation might be, at least, an accelerating factor of intussusception by increased peristalsis. On the ground of clinical course in our case, whole gut irrigation as a bowel preparation should be used with caution because it may accelerate

intussusception.

Early diagnosis and appropriate treatment are essential for intussusception because the mesentery of the involved segment is trapped between the overlapping layers of the bowel and its vascularity may be compromised. Unfortunately, a precise preoperative diagnosis is established in only less than half of the cases^[3,5,11]. Two factors may be responsible for the low accuracy of the preoperative diagnosis in adult intussusception. One reason is the rare incidence of the intussusception itself and the other reason is the vague or nonspecific symptomatology. This condition is often misdiagnosed as a large polyp^[9,12] or rectal prolapse^[7,8] because of the wide variety of nonspecific symptoms that include abdominal pain, nausea and/or vomiting. It is noteworthy that, in most reports, the experience of the adult intussusception at each institution is limited to one or two cases per year^[3-6,11]. Accordingly, the surgeon must keep in mind the possibility of intussusception even though the clinical signs of the patient may be very subtle.

Radiologic investigations may be helpful to distinguish intussusception from other common causes of intestinal obstruction, and for precisely identifying the etiologies of the intussusception preoperatively^[13]. The characteristic finding of intussusception on a contrast study is a cup-shaped filling defect that is often accompanied with an additional filling defect representing the leading tumor^[3]. Hydrostatic reduction under radiologic control, as employed in children, is less effective in adults and risk of perforation during the procedure is not negligible^[10]. Generally, CT scan is acknowledged to be the most useful radiologic method that may provide additional preoperative information including the possible extension and/or dissemination of a malignant tumor^[2,5,11,14]. The most common CT finding is a thickened segment of bowel with an eccentrically placed crescent-like fatty area; this represents the intussusception and the intussuscepted mesentery. This appears either as a round target mass or as a long sausage-shaped mass^[14].

The appropriate management of adult intussusception is not always clear cut. Most authors agree that operative management of adult intussusception is almost always indicated because of high likelihood of neoplasm as a leading point, particularly if this occurs in the colon. There is controversy about the reduction before resection in cases of sigmoidorectal intussusception, even though most surgeons agree that primary surgical resection without a prior attempt at reduction is the treatment of choice in colocolic adult intussusception^[3,5,13]. Several aspects to consider for reduction prior to resection are the reduction of externally viable bowel despite mucosal necrosis, intraluminal or transperitoneal seeding, venous embolization of malignant cells, and spillage of succus through inadvertent perforation^[5]. For patients with sigmoidorectal intussusception, however, the decision regarding the operative procedure may be changed according to tumor involvement of the lower rectum. If the lower rectum is involved, an abdominoperineal resection is indicated. However, without evidence of distal disease, an initial reduction may permit a sphincter-saving procedure instead of the abdominoperineal resection as

advocated by Matsuda *et al*^[15]. In this sense, we would like to emphasize the significance of an attempt at manual reduction of the sigmoidorectal intussusception prior to resection, especially for those cases caused by benign lesion whereby an unnecessary abdominoperineal resection can be avoided. Because most colonic intussusceptions do not lead to complete obstruction, adequate preoperative bowel preparation is generally possible and this allows for a primary anastomosis^[4].

There are good reasons for laparoscopic anterior resection in this case. The first reason is that the wide resection of intussuscepted colon and its mesentery dictates the most important technical consideration, because two-thirds of colocolic intussusceptions are associated with a malignant lesion^[4]. Although preoperative findings were compatible to benign lesion, definite histopathologic diagnosis could not be made in an emergency situation. Moreover, the size of the sessile polyp was large enough (2 cm×2.5 cm on pathologic measurement) for surgeons to consider the possibility of a malignant polyp. In these emergency circumstances, choice of surgical procedure should be judged assuming a possibility of malignancy. The second is the danger in the reduction of externally viable bowel with mucosal necrosis. The third is that intra-abdominal exploration is possible to exclude dissemination in benign-appearing but malignancy-undeniable polyp in an emergency situation. Validities of laparoscopic oncologic colon surgery has been already established in the randomized multicenter trial^[16].

In summary, the sigmoidorectal intussusception caused by sigmoid villotubular adenoma can be reduced by the push-up reduction technique, and safely treated by laparoscopic anterior resection. Our own technique using the sponge-on-the-stick is very useful in the situation that an abdominoperineal resection is, at first, deemed to be inevitable because the intussuscepted segment is very close to the anus. Therefore, this prior reduction technique followed by a resection can be considered for the sigmoidorectal intussusception to avoid inadvertent abdominoperineal resection. In conclusion, our principle of prior reduction technique followed by a laparoscopic resection may be of use in the management of benign sigmoidorectal intussusception involving lower rectum to avoid permanent stoma. Besides, it may also be applicable

to instances of proven malignant intussusception involving the rectum provided that the lesion is confirmed to be localized without intra-abdominal dissemination or metastasis on the laparoscopic exploration before reduction and bowel resection.

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CASE REPORT

Spontaneous resolution of systemic sarcoidosis in a patient with chronic hepatitis C without interferon therapy

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Abstract

A 39-year-old male patient complaining of bilateral hand joint arthralgia was evaluated and found to have chronic hepatitis C and systemic sarcoidosis involving lung, skin, liver, and spleen. Hepatic and cutaneous sarcoidoses were confirmed by the presence of numerous noncaseating granulomas on histological examination. Pulmonary and splenic involvements were diagnosed by imaging studies.

Fifteen months later, the sarcoidotic lesions in lung, liver, and spleen were resolved by radiological studies and a liver biopsy showed no granuloma but moderate to severe inflammatory activity. Systemic sarcoidosis is a rare comorbidity of chronic hepatitis C which may spontaneously resolve.

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Key words: Sarcoidosis; Hepatitis C; Spontaneous resolution

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INTRODUCTION

Sarcoidosis is a granulomatous disorder of unknown etiology, and is characterized by widespread noncaseating granulomas in many organs. Most commonly sarcoidosis affects the lungs, followed by mediastinal, hilar lymph nodes, and skin. Hepatic involvement of systemic

sarcoidosis is frequently demonstrated^[1]. In fact, sarcoidosis is the most common cause of hepatic granulomas in the West, and infectious disorders resulting from HCV or HIV infections are possible comorbidities of hepatic sarcoidosis. Most reported cases of HCV associated-sarcoidosis are not the consequence of HCV infection *per se*, but are the results of interferon antiviral therapy^[2,3]. However, cases of HCV associated-sarcoidosis without interferon therapy have also been recently reported and the possibility of HCV infection *per se* inducing sarcoidosis by activating host immune systems has been suggested^[3,4].

Active sarcoidosis and active viral hepatitis are somewhat incompatible because sarcoidosis is a reflection of activated host cellular immunity to an unknown stimulant, while an augmented cellular immunity is one of the main mechanisms by which HCV replication is suppressed by antiviral agents. Thus, changes in the activities of viral hepatitis *vs* sarcoidosis are worth observing when they simultaneously affect a patient.

We experienced a case of systemic sarcoidosis in a patient with chronic hepatitis C who did not receive interferon therapy, and observed the spontaneous resolution of sarcoidotic lesions with more aggravated hepatitis activities during the natural course of this rare comorbidity.

CASE REPORT

A 39-year-old male patient was admitted because of bilateral hand joint arthralgia, who developed suddenly and impaired hand grip. His serum rheumatoid factor titer (102.7 kU/L) was elevated but other associated clinical features were not compatible with a diagnosis of rheumatoid arthritis. Moreover, his joint symptom improved in 2 d without specific medication.

The patient had suffered from diabetes and chronic hepatitis C for 20 years and received treatment with oral hypoglycemic agents and subcutaneous insulin injections. He was told that his hepatitis activities were mild and need not receive antiviral therapy for chronic hepatitis C. He was in a relatively good condition until 4 mo prior to admission, when he developed fatigue, myalgia, and a mild dry cough. He visited other hospitals for these symptoms and was found to have small pulmonary nodules, which were attributed to past tuberculous infection. His respiratory symptoms improved over the intervening 4 mo, but fatigue and weakness persisted.

A physical examination disclosed no remarkable findings except for three oval brownish papular skin rashes

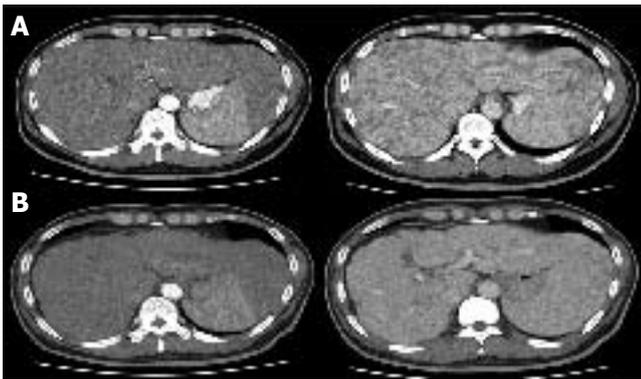


Figure 1 Multiple low-attenuating nodular lesions in the liver and an enlarged spleen (A) and their disappearance after 15 mo (B) on CT images.

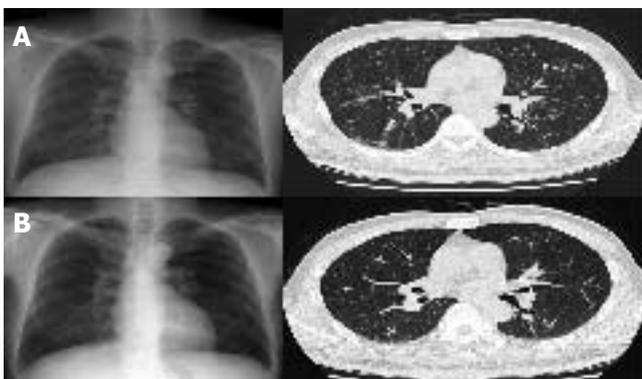


Figure 3 Scattered reticulonodular opacities and multiple fine parenchymal nodules in the case of sarcoidosis (A) and their absence after 15 mo (B).

on the periumbilical and lower back areas. Chest X-ray revealed tiny nodules scattered throughout the lung fields and maxillary sinus haziness was found on skull X-ray. Complete blood cell counts with differential counts were all within the normal ranges. Serological markers for HBV infection (HBsAg and anti-HBs) were negative but anti HCV antibody was positive. A sensitive polymerase chain reaction (PCR) detected serum HCV RNA and identified the genotype of the HCV as 1b. Blood chemistry tests revealed 23 IU/L aspartate aminotransferase (AST), 29 IU/L alanine aminotransferase (ALT), 237 U/L alkaline phosphatase, 8.3 g/dL total protein, 3.8 g/dL albumin, 0.8 mg/dL total bilirubin, 9.4 mg/dL total calcium, 3.0 mg/dL phosphorus, 7.6% hemoglobin A1C, 201 mg/dL fasting blood sugar, 90% (11.6 s, INR 1.05) prothrombin time, 3.3 ng/mL alpha-fetoprotein, 17.3 mg/dL BUN, 0.9 mg/dL creatinine, 1.34 mg/dL free T4 (normal: 0.9-1.8 mg/dL), and 2.84 mIU/L TSH (normal: 0.3-6.5 mIU/L). Cryoglobulin was negative and serum angiotensin converting enzyme concentration was in the upper normal range, 51.8 U/L (normal: 8-52 U/L).

Abdominal ultrasonography showed multiple ill-defined low echoic nodular lesions in the liver and an enlarged spleen implying the presence of liver cirrhosis and hepatocellular carcinoma, but no evidence of enhancement was identified among the numerous low-attenuation nodules scattered throughout the liver and spleen on his

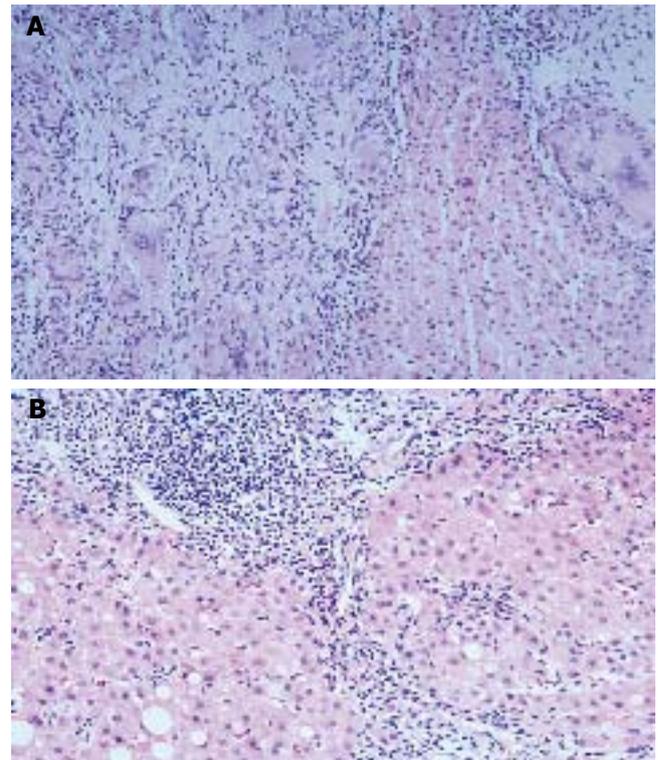


Figure 2 Broad areas of granulomatous inflammation (A) and their disappearance (B) with portal and periportal inflammation, and porto-portal fibrous septa accompanying piecemeal necrosis in hepatic lobules after 15 mo (HE $\times 200$).

abdominal CT scan (Figure 1A). Upper gastrointestinal endoscopic findings were normal and there was no clinical evidence of portal hypertension. A liver biopsy was performed as a basal evaluation of the chronic HCV infection and as a diagnostic procedure for the multiple hepatic nodules, which revealed numerous granulomas with multinucleated giant cells and occasional asteroid bodies in addition to the features of chronic hepatitis such as a mild inflammatory reaction and moderate fibrosis (Figure 2A). Special stains for tuberculosis and fungus, using acid fast bacillus (AFB) and periodic acid Schiff (PAS) were negative, and Mycobacterium tuberculosis nucleic acid was not detected by PCR in the liver tissue.

A high-resolution chest CT scan showed multiple fine nodules distributed in peribronchial areas, especially in the upper and middle lobes, without lymph node enlargement, which was compatible with type III pulmonary sarcoidosis (Figure 3A). Lung tissues obtained by transbronchial lung biopsy revealed a patchy distribution of mild interstitial and perivascular fibrosis, without distinctive granulomas or significant inflammatory cell infiltrations, and stains for AFB and PAS and PCR was negative for Mycobacterium tuberculosis nucleic acids. An analysis of cell types obtained by bronchoalveolar lavage showed that 42% were lymphocytes that consisted of CD4+ cells (63%) and CD8+ cells (37%), a CD4/CD8 ratio of 1.7. Cytologic analysis of bronchial washing fluid for malignant cells was negative and his pulmonary function test was normal.

Granulomatous inflammation with asteroid bodies and multinucleated giant cells were also found in skin biopsy specimens of the periumbilical lesions. Echocardiographic

and ophthalmologic evaluations and a thyroid function test, performed to determine the presence of indolent organ involvement, were all within the normal ranges. With a diagnosis of systemic sarcoidosis involving lung, liver, skin, spleen, and mild chronic hepatitis C, no specific therapies for either sarcoidosis or chronic hepatitis C were adopted, because there was no definitive evidence for significant organ dysfunction and disease progression.

Radiologic and biochemical examinations after 2 months showed no significant changes. After then, he went abroad and was not followed up for a year. Fifteen months later, the hepatosplenic and pulmonary sarcoidotic lesions were remarkably improved on radiologic examination (Figures 1B and 3B) and a liver biopsy showed no remnant granulomas, but moderate to severe inflammatory activities in the lobular and periportal areas, and grade 3 septal fibrosis (Figure 2B). Blood chemistry showed a markedly elevated serum transaminase level (AST 392 IU/mL, ALT 608 IU/mL) with a high serum viral load (serum HCV RNA 2.6×10^5 IU/mL).

DISCUSSION

Liver involvement in sarcoidosis is reported in 40%-70% of patients, but significant hepatic dysfunction is rare^[5]. The histologic features of hepatic sarcoidosis are variable. Granulomas are identified in all the patients and cholestasis presenting as ductal or periductal inflammation and ductopenia are evident in more than 50% of patients. Necroinflammatory changes and vascular changes are found in 41% and 29%, respectively, and hepatic fibrosis is also found in about 20% of patients^[6]. Asteroid bodies are found in about 10% of hepatic sarcoidosis cases^[11]. The hepatic histologic findings in the present case are compatible with those of hepatic sarcoidosis, although chronic HCV infection may also have contributed. Hypodense nodular lesions in the liver and spleen as seen in the CT scans of this patient have been reported to be a characteristic feature of hepatosplenic sarcoidosis^[7]. Splenic sarcoidosis appears as a nonspecific splenomegaly with retroperitoneal lymphadenopathies in most cases of abdominal sarcoidosis, and approximately 15% of cases present as multiple focal low-attenuating nodules^[8,9]. The constitutional symptoms, arthritis, paranasal sinusitis, pulmonary nodules, and noncaseating granulomas in the liver and skin tissues of this patient, are all consistent with the characteristic features of systemic sarcoidosis.

Reported comorbidities of sarcoidosis include infectious diseases, neoplastic disorders, and immunologic-inflammatory diseases such as lupus erythematosus, myasthenia, and primary biliary cirrhosis. HCV infection is reported to be the most commonly associated infectious disease of sarcoidosis, however most cases are associated with interferon antiviral therapy^[2,3]. Treatment-associated cases of sarcoidosis have been reported in many disorders such as chronic hepatitis C, chronic myelogenous leukemia, renal cell carcinoma and lymphoma, in which the beneficial effect of interferon therapy has been established. Importantly, in cases of chronic hepatitis C, interferon can both induce sarcoidosis^[10] and reactivate sarcoidosis^[11], and

interferon-based combination antiviral regimens cannot eliminate the occurrence of sarcoidosis^[12].

Interferon not only has direct antiviral activity but also has potent immune stimulating activities especially on T helper (Th1) immune response^[10,13], which is also involved in the pathogenesis of sarcoidosis^[14,15]. Granulomas in sarcoidosis have an abundance of CD4+ T lymphocytes and mononuclear phagocytes, which are thought to be a result of cytokine stimulation and immunologic dysregulation^[16]. Moreover, ribavirin, an antiviral agent which augments the anti-HCV effect of interferon in chronic hepatitis C, also enhances Th1 cytokine response, while inhibiting Th2 cytokine response^[17,18]. Thus it appears that treatment-associated sarcoidosis in chronic hepatitis C is mediated by an augmentation of Th1 immune response.

Cases of sarcoidosis and chronic hepatitis C with no history of interferon therapy are rarely reported. Bonnet *et al*^[4] reported two cases of pulmonary sarcoidosis associated with untreated chronic hepatitis C, one presented with respiratory and cutaneous symptoms that responded to corticosteroid therapy, the other manifested as cervical lymph adenopathies with pulmonary symptoms and responded poorly to corticosteroids. However, sarcoidosis hepatic involvement was absent in both cases, liver biopsies showed features of chronic hepatitis but no granulomas. Our case is unique in that treatment-unrelated hepatic sarcoidosis and its spontaneous resolution were proven histologically in this chronic hepatitis C patient.

Interferon-based antiviral therapy is the standard treatment for chronic hepatitis C, but careful evaluations are required before starting the treatment because many adverse events can be induced by interferon. Flu like symptoms, cytopenia, depression, hyperglycemia, and thyroid dysfunctions are commonly encountered during interferon therapy. Interstitial lung disease, cardiomyopathy, and retinopathy rarely develop. Thus severe depression, autoimmune disorders, and uncontrolled diabetes contraindicate interferon therapy^[19]. In addition to these disorders, sarcoidosis must also be considered, not only during interferon therapy but also before antiviral therapy, since sarcoidosis may preclude interferon administration.

The clinical course of pulmonary sarcoidosis is variable with a spontaneous remission rate of 40% over a 6-mo observation period before steroid therapy^[20]. In addition, the clinical course of treatment-associated sarcoidosis in chronic viral hepatitis is also variable. The discontinuation of interferon with or without corticosteroid therapy usually improves sarcoidotic lesions^[11], but the remission of pulmonary sarcoidosis has also been reported though interferon therapy is continued^[21]. However, the natural course of treatment-unrelated sarcoidosis in chronic hepatitis C has not been previously reported.

Although corticosteroid therapy can suppress the active inflammatory reaction of sarcoidosis, its effect on clinical outcome has not been fully established. Moreover, it often provokes serious adverse events, such as hepatic functional deterioration and enhanced viral replication^[22]. In a few cases of hepatic sarcoidosis, corticosteroid therapy could normalize liver enzymes but could not achieve histologic improvements^[23-25]. Due to the potential provocation of viral replication and uncertain efficacy in a

clinically stable condition, the patient was simply observed without corticosteroid therapy

In view of the fact that sarcoidosis is a manifestation of an augmented (Th1) immune response to an unidentified stimulant and that interferon-induced immune activation is an important mechanism of viral replication suppression in chronic hepatitis C, hepatitis activities may be suppressed when chronic hepatitis C is combined with active sarcoidosis. In fact, the present case provides clinical evidence that supports this postulation, because serum ALT levels and histologic hepatitis activities were mild when the patient initially presented with active systemic sarcoidosis; whereas 15 mo later when the sarcoidotic lesions resolved, hepatitis activities were aggravated with a higher serum ALT level and a high serum HCV RNA titer. In conclusion, it is prudent to keep in mind that systemic sarcoidosis is a rare comorbidity of chronic hepatitis C which may spontaneously resolve.

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CASE REPORT

Spontaneous chylous peritonitis mimicking acute appendicitis: A case report and review of literature

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Abstract

Acute abdominal pain with signs and symptoms of peritonitis due to sudden extravasation of chyle into the peritoneal cavity is a rare condition that is often mistaken for other disease processes. The diagnosis is rarely suspected preoperatively. We report a case of spontaneous chylous peritonitis that presented with typical symptoms of acute appendicitis such as intermittent fever and epigastric pain radiating to the lower right abdominal quadrant before admission.

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Key words: Acute abdominal pain; Chyle; Appendicitis

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INTRODUCTION

Chylous ascites is the accumulation of a milky or creamy peritoneal fluid rich in triglycerides due to the presence of lymph. It is a rare clinical condition that results from the disruption of the abdominal lymphatic system. Multiple causes have been described including abdominal malignancy, cirrhosis, inflammation, congenital and postoperative or traumatic causes, and miscellaneous disorders. Management is based on identifying and treating the underlying cause. A sudden outpouring of chyle into the peritoneal cavity may produce acute chylous peritonitis. Patients with such defect usually present with

the features of acute abdomen. Very few cases of acute chylous peritonitis have been described in the literature. We have discussed the clinical features and management of acute chylous peritonitis, and its rarity and presentation compared to common surgical emergencies, such as acute appendicitis.

CASE REPORT

A 22-year-old male presented with intermittent fever and abdominal pain for two days before admission. He first presented at a clinic, where acute appendicitis was suspected. He was referred by the clinic to our hospital for further management. The symptoms of abdominal distension and pain were continuous and aggravated by any movement. The patient did not have any history of previous trauma or similar pain, and there was nothing relevant in his past medical history. He did not smoke cigarettes or consume alcohol, and denied any recent long distance or overseas travel. On examination, his temperature was 38.2 °C, pulse rate was 80/min, and blood pressure was 116/70 mmHg. The systemic examination was unremarkable. The abdomen was soft and ovoid in shape, and there was tenderness over the periumbilical and right lower quadrant regions. Bowel sounds were audible. Blood analysis gave the following values: total leukocyte count, $11.6 \times 10^9/\mu\text{L}$; Hb, 143 g/L; platelet count, $31.4 \times 10^9/\mu\text{L}$; neutrophils, 64.3%; lymphocytes, 22.7%; Na, 141 mmol/L; K, 3.9 mmol/L; urea, 3.9 mmol/L; creatinine, 70.7 $\mu\text{mol/L}$; glucose, 5.3 mmol/L; AST, 367 nkat/L; and ALT, 133 nkat/L.

The chest X-ray was normal, but the abdominal X-ray showed nonspecific gas-filled loops of the large intestine. Computed tomography of the abdomen with contrast showed lobulated fluid accumulation over the retroperitoneal space extending from the level of the renal hilum into the right side pelvis and right inguinal area (Figure 1). Suspecting a ruptured appendix, we performed an exploratory laparotomy. When entering the peritoneum, we noted a large amount of milky fluid in the peritoneal cavity. Chylous ascites was first considered because of the obvious lymphatic leakage from the thoracic duct after we opened the retroperitoneum (Figure 2). We performed suture ligation of the lymphatic leakage and drainage of the retroperitoneal lymphocele. Postoperative drainage was < 100 mL in the first week. The patient commenced a low-fat diet, and was recovering well after a 6-month follow-up.

DISCUSSION

Ascites is the presence of excess fluid in the peritoneal



Figure 1 Accumulation of lobulated fluid over the retroperitoneal space and at the para-cecum region shown by computed tomography with contrast to the abdomen.



Figure 2 Obvious lymphatic leakage from the thoracic duct after the retroperitoneum was opened.

cavity and is a common clinical finding with a wide range of causes, which can require varied treatment. Acute chylous peritonitis is defined as an acute abdomen with all the signs of acute peritoneal irritation resulting from free chyle in the peritoneal cavity, without any underlying disease. Acute chylous peritonitis can also involve extravasation of milky or creamy peritoneal fluid that is rich in triglycerides with small amounts of cholesterol and phospholipids, caused by the presence of thoracic or intestinal lymph in the abdominal cavity. Chylous ascites occurs in one in 20 000 patients admitted to the hospital^[1].

The lymphatic system is an accessory route through which fluids and protein flow from the interstitial spaces to the vascular system. Almost all tissues of the body have lymphatic channels composed of one-way valves that drain the excess fluid from the interstitial spaces of tissue. These channels play a pivotal role in clearing the interstitium of debris and bacteria, which are carried to lymph nodes where they are opsonized and phagocytized. Gastrointestinal tract lymphatics also transport absorbed water and lipids to the circulatory system^[2]. In the gut, long chain triglycerides are converted into monoglycerides, free fatty acids, and absorbable chylomicrons, which explain

the high content of triglycerides and the milky, cloudy appearance of lymph. In our case, the triglyceride concentration in the ascites fluid was 5.48 mmol/L, much higher than the blood triglyceride concentration of 102 mg/dL. The basal flow rate of lymphatic fluid through the thoracic duct averages about 1.0 mL/kg per an hour, with a total volume of 1 500 - 1 700 mL/d. These volumes increase markedly with the ingestion of fats, and fluid rates as high as 200 mL/h have been reported^[3].

Chylous ascites may result from many pathological conditions, including congenital defects of the lymphatic system; nonspecific bacterial, parasitic, and tuberculous peritoneal infections; liver cirrhosis; malignant neoplasm; and blunt abdominal trauma. These etiologies may be categorized into distinct mechanism-based groups^[4]. The most common etiological factors are abdominal malignancy and congenital lymphatic abnormalities in adults and children, respectively^[5]. We have presented an unusual case of spontaneous chylous ascites-related peritonitis mimicking acute appendicitis in a young adult. Abdominal surgery is another common cause that is most frequently associated with the resection of an abdominal aortic aneurysm or retroperitoneal lymph node dissection^[6]. Various vascular surgical procedures such as aorta-to-femoral artery bypass may cause chylous complications^[7]. It has been suggested that overloading of the lymphatic channels with chyle after a heavy fatty meal may cause extravasation of chyle intraperitoneally and retroperitoneally^[8].

Abdominal distension is the most common symptom in patients with chylous ascites. Other clinical features include abdominal pain, anorexia, weight loss, edema, weakness, nausea, dyspnea, weight gain, lymphadenopathy, early satiety, fever, and night sweats. The clinical presentation of acute abdomen is less common. Free chyle is relatively nonirritating to the serosal surface, but pain may result from the stretching of the retroperitoneum and the mesenteric serosa^[8]. The pain is severe and a physical examination may lead to the misdiagnosis of appendicitis, cholecystitis, mesenteric arterial embolism, or a perforated viscus. The unusual case we present here is spontaneous chylous ascites-related peritonitis mimicking acute appendicitis in a young adult. Just like a similar case reported by Lamblim *et al*^[9] the diagnosis was established by the celiotomy.

The diagnosis of chylous ascites is confirmed by analyzing the ascites fluid, which is only possible if such a diagnosis is suspected preoperatively. The chief characteristics of chylous effusions include a milky appearance, separation into a creamy layer on standing, lacking an odor, alkaline chemical properties, specific gravity greater than 1.012, bacteriostatic properties, 3% total protein, staining of fat globules with Sudan red stain, fat content of 0.4% - 4%, and total solids > 4%^[10]. The triglyceride level is an important diagnostic tool, and concentration in the chylous ascites is typically two to eight times that of plasma^[11]. True chylous ascites must be distinguished from "chyliform" and "pseudochylous" effusions, in which the turbid appearance is due to cellular degeneration from bacterial peritonitis or neoplasm.

However, the triglyceride concentration is low in these effusions. Other diagnostic tests such as computed tomography, lymphangiography, lymphoscintigraphy, and laparotomy have the highest yield of diagnostic information^[4].

The optimal management of true chylous peritonitis depends upon the underlying etiology. In patients with symptoms of an acute abdominal process, immediate exploration should be performed. Laparotomy usually allows a definitive diagnosis and provides an opportunity to address the underlying cause. The source of chylous extravasation can be corrected by ligation of the leaking lymphatics or removal of the offending lesion, the cause of many congenital and all traumatic cases. The goals of nonsurgical therapy for chylous ascites include maintaining or improving nutrition, and decreasing the rate of chyle formation. Dietary intervention involves a diet that is rich in protein, and low in fat and medium-chain triglycerides to decrease lymph flow in the major lymphatic tracts and to facilitate the closure of chylous fistulas^[12]. Total parenteral nutrition can be used to achieve complete bowel rest and might allow resolution of the chylous ascites. Somatostatin improves chylous ascites by inhibiting lymph fluid excretion through specific receptors found in the normal intestinal wall of lymphatic vessels^[13]. In patients with a large amount of ascites, a total paracentesis to relieve discomfort and dyspnea can be performed and repeated as needed. However, one should note the risk of infection and fat emboli. If the patients are poor surgical candidates and refractory to nonsurgical treatment, peritoneovenous shunting may be an option, although these shunts carry a high rate of complications^[14].

In conclusion, we have described a rare case of acute chylous peritonitis that mimicked acute appendicitis, but could not identify the cause. This case suggests that diagnostic laparoscopy may play an important role in the initial management of this condition^[15].

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Percutaneous local therapies for hepatocellular carcinoma impair gastric function

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TO THE EDITOR

Percutaneous local therapies, such as percutaneous ethanol injection (PEI), microwave coagulation and radiofrequency ablation (RFA), are frequently used worldwide for the treatment of hepatocellular carcinoma (HCC) because of their high effectiveness. Although these treatment modalities can induce effectively coagulated tumor necrosis in the liver, they may cause adverse effects on extrahepatic abdominal organs. There are, however, no published reports on the influence of percutaneous local therapies on the gastric myenteric activity. Therefore, it is unclear whether or not gastric function is affected by percutaneous local therapies. In this study, to make clear the effect of PEI and RFA on the gastric function, we continuously recorded the gastric myoelectric activity by electrogastrography (EGG) and estimated the effect of percutaneous local therapies for HCC on gastric function.

Five patients with HCC (3 males and 2 females; age

ranging from 66 to 81 years) were enrolled in the present study. Written informed consent was obtained from each patient. We have developed several novel percutaneous local therapies for HCC. The first is the combination of PEI and RFA (PEI-RFA). In this treatment modality, RFA is performed immediately after PEI. The second is percutaneous ethanol-lipiodol injection (PELI). In this modality, mixture of pure ethanol and lipiodol, a lipid-based contrast medium, at a ratio of 10:1, is injected percutaneously into HCC. The last is the combination of PELI and RFA (PELI-RFA). Usefulness of these new treatment modalities has been reported elsewhere^[1-7]. In the present study, two patients with HCC underwent PELI and three underwent PELI-RFA.

We recorded EGG before and 3 d after therapy, and the results were compared. EGG was recorded with a portable electrogastrographic recorder (NIPRO; Tokyo, Japan). Five electrodes were affixed to the abdomen as shown in Figure 1, and EGG was recorded for 30 min during a fasting period and again during a postprandial period. We evaluated the percentages of bradygastria (<2.4 c/min), normogastria (2.4 - 3.6 c/min), and tachygastria (>3.6 c/min), as well as the dominant frequency (DF) and the postprandial-to-fasting power ratio (PR). We also examined clinical abdominal symptoms, using the questionnaire reported by Svedlund *et al.* (Gastrointestinal Symptom Rating Scale, GSRS)^[8] which was translated into Japanese. The translated form was provided by Astra Zeneca (Tokyo, Japan). Measured values were expressed as mean ± SE. Comparisons before and after therapy were performed by the paired Student's *t* test, and *P* < 0.05 was accepted as a significant difference.

The results are summarized in Table 1. Because the similar EGG patterns were obtained from all the channels, the data of channel 1 are shown in Table 1. After percutaneous local therapies for HCC, the percentages of bradygastria in the fasting period were significantly increased, while the percentages of normogastria in the same period were significantly decreased. The PR of normogastria and bradygastria was significantly decreased after therapy. Conversely, no significant differences were found in the calculated GSRS scores obtained from the questionnaire before and after therapy. This study is the first report to estimate the effect of percutaneous local therapies for HCC on the gastric myenteric activity. The present results indicate that percutaneous therapies for HCC may impair gastric function even when clinical symptoms are not apparent.

The first EGG measurement in humans was performed by Alvarez^[9]. This method can be used to noninvasively assess the electrical activity generated by gastric smooth

Table 1 Comparisons between before and after therapy (mean ± SE)

| | Before | After |
|---------------------------|------------|-------------------------|
| Bradygastria (%) | 54.0 ± 7.4 | 77.4 ± 5.6 ^a |
| Normogastria (%) | 42.4 ± 7.5 | 18.6 ± 5.2 ^a |
| Tachygastria (%) | 3.2 ± 1.8 | 4.0 ± 4.0 |
| PR of bradygastria (%) | 1.3 ± 0.1 | 0.8 ± 0.1 ^a |
| PR of normogastria (%) | 2.5 ± 0.5 | 1.8 ± 0.5 ^a |
| PR of tachygastria (%) | 2.1 ± 0.5 | 1.7 ± 0.5 |
| DF (c/min) | 2.3 ± 0.1 | 1.9 ± 0.1 |
| Gastrointestinal symptoms | 1.8 ± 0.2 | 2.2 ± 0.5 |
| Total score | | |

^a*p* < 0.05 vs before therapy

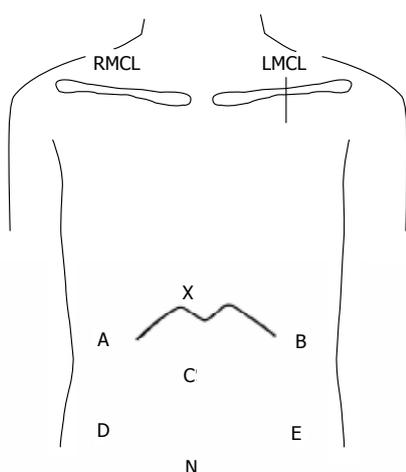


Figure 1 The positions of electrodes for EGG recording. X, xiphoid process; N, navel; RMCL, right mid-clavicular line; LMCL, left mid-clavicular line; C, central terminal electrode placed on the patient's ventral midline about halfway between the umbilicus and the xiphoid process; A, channel 1 placed on an intersecting point between RMCL and a vertical bisectrix of the line XC; B, channel 2 placed on intersecting point between LMCL and a vertical bisectrix of the line XC; D, channel 3 placed on intersecting point between RMCL and a vertical bisectrix of the line NC; E, channel 4 placed on intersecting point between LMCL and a vertical bisectrix of the line NC.

muscles. EGG is believed to reflect the electrical control activity and gastric motility regulated by pacemakers. In humans, these EGG waves originated from the pacemaker area along the major curvature of the stomach and propagated aborally with increasing velocity, at intervals of approximately 20 s^[10]. EGG has been shown to provide useful information for clinical diagnoses^[10]. EGG abnormalities have been observed in disorders of gastric emptying, nausea and vomiting^[10].

Percutaneous local therapies is of great significance in the treatment of HCC and metastatic liver tumors. PELI, PEI-RFA and PELI-RFA are new therapeutic methods for HCC, which we have developed. We have confirmed the usefulness of these novel percutaneous local therapies in the treatment of HCC^[1-7]. It has been shown that transcatheter arterial chemoembolization affected the gastric myenteric activity and that overproduction of endogenous prostaglandin was related to dysrhythmia of the gastric myenteric activity^[11]. In this pilot study,

we demonstrated that the gastric myenteric activity was affected by percutaneous local therapies for HCC, although abdominal symptoms were not apparent and GSRS scores obtained from the questionnaire did not change significantly after therapy. It is a significant clinical matter that delayed gastric transit may occur after percutaneous therapy for HCC. Because it has been reported that patients with HCC tend to have gastrointestinal dysfunction^[12], we have to pay attention to gastric dysfunction after percutaneous local therapies for HCC even when there are no clinical symptoms. The mechanisms underlying the effect of percutaneous local therapies for HCC on extrahepatic abdominal organs need further exploration.

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- 1 **Das KM**, Farag SA. Current medical therapy of inflammatory bowel disease. *World J Gastroenterol* 2000; 6: 483-489 [PMID: 11819634]
- 2 **Pan BR**, Hodgson HJF, Kalsi J. Hyperglobulinemia in chronic liver disease: Relationships between *in vitro* immunoglobulin synthesis, short lived suppressor cell activity and serum immunoglobulin levels. *Clin Exp Immunol* 1984; 55: 546-551 [PMID: 6231144]
- 3 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaobao Zazhi* 1999; 7: 285-287

Books and other monographs (list all authors)

- 4 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

- 5 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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- 6 **Morse SS**. Factors in the emergence of infectious diseases. *Emerg Infect Dis* serial online, 1995-01-03, cited 1996-06-05; 1(1):24 screens. Available from: URL: <http://www.cdc.gov/ncidod/EID/eid.htm>

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Present as mean \pm SD and mean \pm SE.

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Use SI units. For example: body mass, *m* (B) = 78 kg; blood pressure, *p*(B) = 16.2/12.3 kPa; incubation time, *t* (incubation) = 96 h, blood glucose concentration, *c* (glucose) 6.4 \pm 2.1 mmol/L; blood CEA mass concentration, *p* (CEA) = 8.6 24.5 μ g/L; CO₂ volume fraction, 50 mL/L CO₂ not 5% CO₂; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, etc. Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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Italicis

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

Genotypes: *gyrA*, *arg 1*, *c myc*, *c fos*, etc.

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| Number | Nonstandard | Standard | Notice |
|--------|--|--|--|
| 1 | 4 days | 4 d | In figures, tables and numerical narration |
| 2 | 4 days | four days | In text narration |
| 3 | day | d | After Arabic numerals |
| 4 | Four d | Four days | At the beginning of a sentence |
| 5 | 2 hours | 2 h | After Arabic numerals |
| 6 | 2 hs | 2 h | After Arabic numerals |
| 7 | hr, hrs, | h | After Arabic numerals |
| 8 | 10 seconds | 10 s | After Arabic numerals |
| 9 | 10 year | 10 years | In text narration |
| 10 | Ten yr | Ten years | At the beginning of a sentence |
| 11 | 0,1,2 years | 0,1,2 yr | In figures and tables |
| 12 | 0,1,2 year | 0,1,2 yr | In figures and tables |
| 13 | 4 weeks | 4 wk | |
| 14 | Four wk | Four weeks | At the beginning of a sentence |
| 15 | 2 months | 2 mo | In figures and tables |
| 16 | Two mo | Two months | At the beginning of a sentence |
| 17 | 10 minutes | 10 min | |
| 18 | Ten min | Ten minutes | At the beginning of a sentence |
| 19 | 50% (V/V) | 500 mL/L | |
| 20 | 50% (m/V) | 500 g/L | |
| 21 | 1 M | 1 mol/L | |
| 22 | 10 μM | 10 μmol/L | |
| 23 | 1N HCl | 1 mol/L HCl | |
| 24 | 1N H ₂ SO ₄ | 0.5 mol/L H ₂ SO ₄ | |
| 25 | 4rd edition | 4 th edition | |
| 26 | 15 year experience | 15- year experience | |
| 27 | 18.5 kDa | 18.5 ku, 18 500u or M:18 500 | |
| 28 | 25 g.kg ⁻¹ /d ⁻¹ | 25 g/(kg·d) or 25 g/kg per day | |
| 29 | 6900 | 6 900 | |
| 30 | 1000 rpm | 1 000 r/min | |
| 31 | sec | s | After Arabic numerals |
| 32 | 1 pg L ⁻¹ | 1 pg/L | |
| 33 | 10 kilograms | 10 kg | |
| 34 | 13 000 rpm | 13 000 g | High speed; g should be in italic and suitable conversion. |
| 35 | 1000 g | 1 000 r/min | Low speed. g cannot be used. |
| 36 | Gene bank | GenBank | International classified genetic materials collection bank |
| 37 | Ten L | Ten liters | At the beginning of a sentence |
| 38 | Ten mL | Ten milliliters | At the beginning of a sentence |
| 39 | umol | μmol | |
| 40 | 30 sec | 30 s | |
| 41 | 1 g/dl | 10 g/L | 10-fold conversion |
| 42 | OD ₂₆₀ | A ₂₆₀ | "OD" has been abandoned. |
| 43 | One g/L | One microgram per liter | At the beginning of a sentence |
| 44 | A260 nm ^b P<0.05 | A ₂₆₀ nm ^a P<0.05 | A should be in italic. In Table, no note is needed if there is no significance instatistics: ^a P<0.05, ^b P<0.01 (no note if P>0.05). If ther is a second set of P value in the same table, ^c P<0.05 and ^d P<0.01 are used for a third set: ^a P<0.05, ⁱ P<0.01. |
| 45 | [*] F=9.87, [§] F=25.9, [#] F=67.4 | ¹ F=9.87, ² F=25.9, ³ F=67.4 | Notices in or under a table |
| 46 | KM | km | kilometer |
| 47 | CM | cm | centimeter |
| 48 | MM | mm | millimeter |
| 49 | Kg, KG | kg | kilogram |
| 50 | Gm, gr | g | gram |
| 51 | nt | N | newton |
| 52 | l | L | liter |
| 53 | db | dB | decibel |
| 54 | rpm | r/min | rotation per minute |
| 55 | bq | Bq | becquerel, a unit symbol |
| 56 | amp | A | ampere |
| 57 | coul | C | coulomb |
| 58 | HZ | Hz | |
| 59 | w | W | watt |
| 60 | KPa | kPa | kilo-pascal |
| 61 | p | Pa | pascal |
| 62 | ev | EV | volt (electronic unit) |
| 63 | Jonle | J | joule |
| 64 | J/mmol | kJ/mol | kilojoule per mole |
| 65 | 10×10×10cm ³ | 10 cm×10 cm×10 cm | |
| 66 | N·km | KN·m | moment |
| 67 | x±s | mean±SD | In figures, tables or text narration |
| 68 | Mean±SEM | mean±SE | In figures, tables or text narration |
| 69 | im | im | intramuscular injection |
| 70 | iv | iv | intravenous injection |
| 71 | Wang et al | Wang <i>et al.</i> | |
| 72 | EcoRI | EcoRI | <i>Eco</i> in italic and RI in positive. Restriction endonuclease has its prescript form of writing. |
| 73 | Ecoli | <i>E.coli</i> | Bacteria and other biologic terms have their specific expression. |
| 74 | Hp | <i>H pylori</i> | |
| 75 | Iga | Iga | writing form of genes |
| 76 | igA | IgA | writing form of proteins |
| 77 | ~70 kDa | ~70 ku | |

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Causal role of *Helicobacter pylori* infection in gastric cancer

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Abstract

Gastric cancer is the second most frequent cancer in the world, accounting for a large proportion of all cancer cases in Asia, Latin America, and some countries in Europe. *Helicobacter pylori* (*H pylori*) is regarded as playing a specific role in the development of atrophic gastritis, which represents the most recognized pathway in multistep intestinal-type gastric carcinogenesis. Recent studies suggest that a combination of host genetic factors, bacterial virulence factors, and environmental and lifestyle factors determine the severity of gastric damage and the eventual clinical outcome of *H pylori* infection. The seminal discovery of *H pylori* as the leading cause of gastric cancer should lead to effective eradication strategies. Prevention of gastric cancer requires better screening strategies to identify candidates for eradication.

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Key words: Gastric cancer; Host genetic factors; *H pylori*; Bacterial virulence factors.

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INTRODUCTION

Helicobacter pylori (*H pylori*) is a Gram-negative, microaerophilic bacterium which expresses an abundant amount of urease. Infection with this bacterium is a worldwide phenomenon. Prevalence increases with age, but differs quite dramatically among populations^[1-4]. In the USA, prevalence is less than 20% at 20 years old and approximately 50% at 50 years^[2]. In Japan, it is less than

20% under 20 years, increasing to a plateau of 70-80% at 40 years^[3], while in Korea, it is 50% at 5 years and 90% at 20 years^[4].

The epidemiological data suggest that *H pylori* gastritis is associated with gastric carcinogenesis^[5,6]. *H pylori* colonizes the gastric mucosa and elicits both inflammatory and immune lifelong responses, including the release of various bacterial and host-dependent cytotoxic substances^[7]. Pathological and clinical studies have convincingly proved the etiological role of *H pylori* in the development of chronic gastritis^[8] and peptic ulcer^[9]. Moreover, *H pylori* infection has been recognized as a risk factor for both the diffuse and intestinal types of gastric cancer^[10], and the bacterium itself is classified as a class I carcinogen by the World Health Organization and International Agency for Research on Cancer Consensus Group^[11].

H pylori strains carrying the cytotoxin-associated gene A (*cagA*) gene are strongly associated with an increased risk of gastric adenocarcinoma^[12]. Recent studies suggest that the severity of gastric damage and eventual clinical outcome of *H pylori* infection are determined by a combination of host genetic and bacterial virulence factors^[13-17]. *cagA* is divided into two major subtypes, the East Asian and Western types^[18]. The grade of gastric atrophy (and therefore gastric cancer risk) is higher in patients with East Asian *cagA*-positive strains than in those with *cagA*-negative or Western *cagA*-positive strains. Of interest is that atrophy grade varies even among patients with East Asian *cagA*-positive strains^[19], and that most *H pylori*-infected subjects in fact develop no significant disease, remaining asymptomatic throughout their lives. The reasons for this are not explained by bacterial virulence factors alone; rather, genetic factors of the host should also be considered to play a role in *H pylori*-induced outcomes.

Here, we discuss recent developments in gene-environment interaction and the importance of *H pylori* eradication in the prevention of gastric cancer.

Association between *H pylori* and gastric cancer

H pylori has been associated with the location of gastric cancers, specifically those of the body and antrum. No association is seen with the location of cardiac tumors^[20]. *H pylori* gastritis is characterized by severe, acute and chronic inflammation which would last for decades if not treated^[21]. Such persistent inflammation likely has serious biological implications. For example, activated neutrophils generate reactive oxygen and nitrogen species, which are mutagenic and carcinogenic^[22,23]. Atrophic

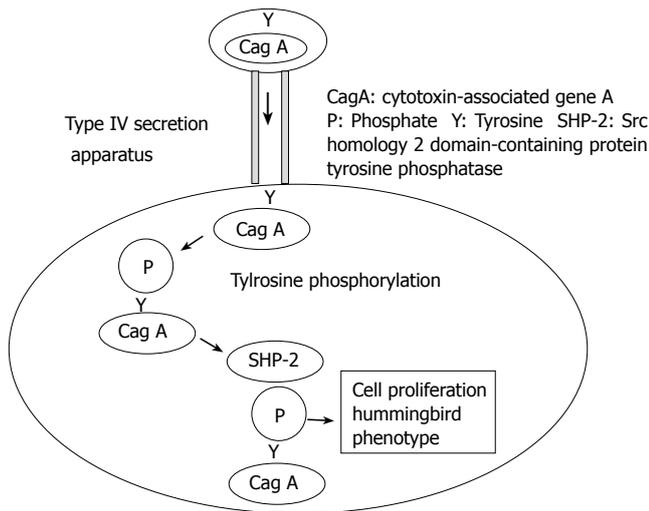


Figure 1 Deregulation of SHP-2 tyrosine phosphatase by the *H. pylori* virulence factor CagA. CagA is delivered into gastric epithelial cells by the bacterial type IV secretion system. CagA deregulates the SHP2 oncoprotein. CagA is noted for its variation, particularly at the SHP2-binding site.

gastritis, intestinal metaplasia and dysplasia are known to be precancerous. Uemura *et al* reported that gastric cancer developed in 36 (2.9%) of 1246 infected and none of 280 uninfected patients^[24]. Among their patients with *H. pylori* infection, those with severe gastric atrophy, corpus-predominant gastritis and intestinal metaplasia were at a significantly higher risk for gastric cancer. Direct associations between *H. pylori* infection and gastric cancer have been examined for many regions and ethnic groups^[25]. In their retrospective analysis of a nested case-control study, Wang *et al* reported that the risk of death from gastric cancer in the *H. pylori* -positive cohort was 1.985 times to the -negative control cohort (95% CI, 1.0026-3.9301), and that the OR of *H. pylori* infection for gastric cancer was 4.467 (95% CI, 1.161-17.190)^[26]. The high incidence of infection in patients with gastric cancer has been confirmed for both the intestinal and diffuse types, and is particularly strong in the former^[27]. However, while *H. pylori* appears to play a role in the initial step as a causative agent for chronic gastritis, the development of gastric cancer is multi-factorial.

Khanna *et al* in India reported a lower prevalence of *H. pylori* infection in gastric cancer patients than in the healthy controls, without significance, and suggested that *H. pylori* may not be responsible for gastric cancer^[28]. On the other hand, Enroth *et al* showed that relative risk estimates for the association between *H. pylori* and gastric cancer risk are to some extent determined by the diagnostic method used to detect *H. pylori* infection^[29]. The loss of serological markers of *H. pylori* infection following the onset of severe atrophy and intestinal metaplasia is a well-known phenomenon, not least in Japan^[30].

While some studies have reported no association with gastric cancer, the summary statistic derived from a meta-analysis indicated no doubt as to the existence of an association^[25].

Role of *cagA*-positive *H. pylori* in the pathogenesis of gastric cancer

Recent studies have suggested that patients infected with *cagA*-positive strains of *H. pylori* are at a significantly higher risk for gastric cancer than those carrying *cagA*-negative strains^[31-36]. *CagA* protein, encoded by the *cagA* gene, is one of the most studied virulence factors of *H. pylori* and is a highly immunogenic protein. The *cagA* gene is one of several genes of a pathogenicity island (PAI) called the *cag* PAI. The *cag* PAI contains 31 genes, 6 of which are thought to be encoded by a putative type IV secretion system. Although *H. pylori* *cagA*-positive isolates from the USA and Japan induce similar IL-8 and apoptosis levels^[37], the grade of gastric atrophy (and gastric cancer risk) is higher in patients with the East Asian *cagA*-positive strains than in those with *cagA*-negative or Western *cagA*-positive strains^[18]. In Asian populations, however, almost all infected subjects harbor *cagA*-positive strains, raising legitimate questions about the relevance of this virulence factor as a risk determinant in such populations.

Held *et al* reported that although patients with antibodies to *CagA* at greatest risk of gastric cancer, risk is still significantly higher in those with *cagA*-negative *H. pylori* infections than in uninfected persons^[38]. Anti-*CagA* responses correlate with neutrophil infiltration, a low anti-*H. pylori* IgG titer or combined with *H. pylori* seronegativity was closely associated with non-cardia gastric cancer, independently of ethnicity^[40]. A meta-analysis of the relationship between *CagA* seropositivity and gastric cancer showed that infection with *cagA*-positive *H. pylori* increased the risk for gastric cancer over that with *H. pylori* infection alone^[41]. Because antibodies to *CagA* remain positive for longer than IgG antibodies to *H. pylori*^[42,43], the risk for gastric cancer based on *H. pylori* IgG antibody might be underestimated^[35,44].

CagA is delivered into epithelial cells by the *cag* type IV secretion system, then phosphorylated on tyrosine residues and wired to the eukaryotic signal transduction pathway, which plays a major role in *H. pylori*-host cell interactions and pathogenesis (Figure 1)^[45-48]. In the injected gastric epithelial cell, *CagA* induces cellular spreading and elongation, termed the hummingbird phenotype, and this is thought to play a crucial role in the pathogenesis of *cagA*-positive *H. pylori* infection. This *CagA*-dependent morphological transformation of gastric epithelial cells requires src homology 2 domain-containing protein tyrosine phosphatase-2 (SHP-2)^[49]. SHP-2 plays a key role in the intracellular signaling elicited by a number of growth factors, hormones and cytokines^[50,51]. East Asian-type *CagA* exhibits stronger SHP-2-binding activity than Western-type *CagA*^[19]. *CagA*-SHP-2 signaling may induce apoptosis and elevate the epithelial cell turnover associated with *cagA*-positive *H. pylori* infection^[26-32]. Extra cycles of DNA replication would increase the chance of genetic mutation leading to abnormal proliferation.

We have reported the association of a frequent single nucleotide polymorphism (SNP, JST057927, G-to-A) in the *PTPN11* gene that encodes SHP-2 with gastric atrophy and gastric cancer^[52]. We found that this polymorphism increased the risk of gastric atrophy and gastric cancer among *H. pylori*-seropositive Japanese subjects. Carriage of the G allele of *PTPN11* increased the risk of atrophy

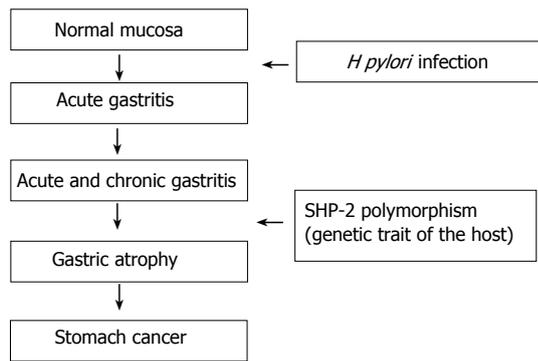


Figure 2 Multistep model for the progression of gastric cancer.

whereas the *A/A* genotype was protective against it. The SHP-2-binding activity of *CagA* influences its virulence in the induction of gastric atrophy, the precursor lesion of gastric cancer. *PTPN11 G/A* polymorphism may constitute a genetic trait of the host predisposing to atrophy among those infected (Figure 2). *CagA*-SHP-2 complex formation may induce abnormal proliferation and movement of gastric epithelial cells, cellular changes that may eventually lead to gastric atrophy and gastric carcinoma. Against this, however, several groups have reported that SHP-2 is not involved in *CagA* action^[53,54]. The resolution of this controversy is awaited.

Effect of pro- and anti-inflammatory cytokine gene polymorphism on *H pylori*-induced gastric cancer

High mucosal levels of cytokines in *H pylori*-infected patients have been reported, including IL-8, IL-6, IL-1B, TNF- α , MIP 1 α and IL-2^[55-62]. Host cytokine gene polymorphisms *IL-1B*, *IL-1RN*, *TNF- α* and *IL-10* are suggested to be part of the genetic background predisposing patients to noncardia gastric cancer in response to *H pylori*^[13,15,17,63]. *IL-1B* and *TNF- α* are functional polymorphisms that affect the production of IL- β and TNF- α , which inhibit gastric acid secretion^[64,65]. The *IL-1B* gene encoding IL-1 β is highly polymorphic, and several diallelic polymorphisms have been reported, two in the promoter region at positions -511 and -31, representing C-T and T-C transitions, respectively. Several studies have shown that these two polymorphisms are in near-total linkage disequilibrium^[13,66]. They have been shown to significantly affect gastric mucosal IL-1 β production in response to *H pylori* infection^[67,68], and it is this higher production of IL-1 β which most likely mediates their effect on gastric acid secretion. Zamboni *et al* have reported that among host genetic factors contributing to *H pylori* disease outcome, *IFN- γ AA* favors *cagA*-positive *H pylori* infection, *TNF- α TT* favors duodenal ulcer, while *IL-10 TT* favors intestinal metaplasia and noncardia gastric cancer^[69]. In Japan and Korea, however, these associations appear less clear^[70,71].

***H pylori*-induced gastric cancer and environmental and lifestyle factors**

A joint World Health Organization/Food and Agriculture Organization Expert Consultation concluded that salt and salt-preserved food probably increase the risk of gastric

cancer^[72]. Substantial evidence from ecological, case-control and cohort studies suggest that cancer risk may also increase with a high intake of some traditional salt-preserved foods and salt *per se*, and that this risk could be decreased with a high intake of fruits and vegetable^[73,74]. Other established non-dietary factors include cigarette smoking^[75]. Tsugane *et al* have documented that the consumption of salted food (pickled vegetables and miso soup) appears to increase the risk of *H pylori* infection^[76]. Salted food intake has been shown to act synergistically to promote the development of gastric cancer in Mongolian gerbils treated with N-methyl-N-nitrosourea (MNU)^[77], and a synergistic enhancing effect between salted food intake and *H pylori* infection has also been reported in a case-control study in Korea^[78]. Motani *et al*^[79] reported that smoking and a high intake of miso soup were associated with noncardia cancer regardless of *H pylori* infection, and also a strong association between *cagA*-positive *H pylori* and noncardia cancer. Although *H pylori* infection is clearly an important risk factor for gastric cancer, smoking cessation and dietary modification may be practical strategies for the prevention of non-cardia gastric cancer among both *H pylori*-positive and -negative subjects.

Conclusive proof for a preventive effect of *H pylori* eradication on gastric carcinogenesis will never be available, because doing so would require the inclusion of individuals in a placebo trial in which the end point is gastric cancer; for not only practical but also ethical and economic reasons, no such study will ever be performed. The alternative is randomized controlled studies that are designed to examine the regression of preneoplastic conditions, such as intestinal metaplasia and gastric atrophy, as surrogate end points of eradication treatment success. One such study is a prospective, randomized, placebo-controlled, population-based primary prevention study of 1630 healthy carriers of *H pylori* infection from Fujian Province, China, recruited in July 1994 and followed up until January 2002^[80]. A total of 988 participants did not have precancerous lesions (gastric atrophy, intestinal metaplasia, or gastric dysplasia) on study entry. Patients were randomly assigned to receive *H pylori* eradication treatment by a 2-wk course of omeprazole 20 mg, a combination product of amoxicillin and clavulanate potassium, 750 mg, and metronidazole 400 mg, all twice daily ($n = 817$); or placebo ($n = 813$). Among the 18 new cases of gastric cancer that developed, no overall reduction was observed in participants who received *H pylori* eradication treatment ($n = 7$) compared with those who did not ($n = 11$). In a subgroup of patients with no precancerous lesions on entry, no patient developed gastric cancer during a follow-up of 7.5 years after *H pylori* eradication treatment compared with those who received placebo (0 vs 6; $P = 0.02$). Although the incidence of gastric cancer development at the population level was similar between participants receiving *H pylori* eradication treatment and those receiving placebo over 7.5 years in a high-risk region of China, eradication of *H pylori* significantly decreased the development of gastric cancer in the subgroup of *H pylori* carriers without precancerous lesions.

A second study was conducted in Hong Kong^[81].

The authors randomized 435 subjects into placebo and eradication groups, the latter of whom received a one-week course of anti-*H pylori* therapy of OAC (omeprazole 20 mg, amoxicillin 1g, and clarithromycin 500 mg twice a day). Clearance of *H pylori* infection at 5 years was confirmed by histology in 164 (74.5%) who had received the eradication therapy versus only 20 (9.3%) subjects in the placebo group. Ten subjects developed invasive gastric cancer during the 5-year follow-up period, four in the eradication and six in the placebo group. Overall progression of gastric intestinal metaplasia (IM), defined as a surrogate marker of cancer, was seen in 52.9% of subjects. Eradication of *H pylori* was significantly associated with a decrease in the risk of IM progression. Patients assigned to receive OAC had a significantly lower risk of progression compared with those who received placebo (OR for progression 0.63; 95% CI, 0.43-0.93). When those in the OAC group with eradication were compared with those in the placebo group with persistent infection, the OR of histological progression was further reduced to 0.48 (95% CI, 0.32-0.74). Although this intervention study failed to demonstrate an effect on gastric cancer risk, eradication of *H pylori* was protective against the progression of a premalignant gastric lesion, namely IM.

Another study was reported from Mexico^[82]. A total of 316 *CagA*-positive subjects were randomized into placebo ($n=155$) and eradication groups ($n=161$), who received 20 mg of omeprazole, 1 g of amoxicillin and 500 mg of clarythromycin, all twice a day for 1 week. Endoscopy was performed at baseline and at 6 weeks and 1 year, with seven biopsies from each endoscopy reviewed by two pathologists. Cure rates in the eradication group were 79.2% and 75.7% at 6 weeks and 1 year, respectively, compared with respective placebo rates of 2.9% and 1.9% ($P<0.001$). Outcome measures were both a consensus "worst biopsy" diagnosis and a weighted index score that incorporated the degree of severity of preneoplasia, with changes in these outcomes compared over time. No significant change in the worst biopsy diagnosis was observed between groups (improvement/worsening: placebo, 19.4%/10.5%; treatment, 22.5%/8.3%; $P=0.74$). The change in index score was favorably greater in the treatment than in the placebo subjects (intention-to-treat analysis, $P=0.03$). These studies of intermediate biomarkers provide circumstantial evidence that *H pylori* eradication diminishes the risk of gastric cancer.

Following partial gastrectomy, the mucosa of the residual stomach usually undergoes severe changes, such as gastric atrophy, intestinal metaplasia and dysplasia^[83,84]. The 1996 Maastricht Consensus Report strongly recommended eradication in infected patients who had undergone gastrectomy for early gastric cancer^[85]. Definite proof of the merit of eradication awaits the completion of a large randomized trial using cancer as the outcome. Nevertheless, the evidence now available supports a conclusion for eradication.

Effective *H pylori* eradication along with a natural decrease in infection due to improved living conditions have resulted in declining gastric cancer rates in Western countries, although this still remains a significant cause of morbidity and mortality in other parts of the world. While

the genes in *cag* PAI are the most strongly virulence-related among those reported to date, other genes have also been reported as candidates that determine outcome in *H pylori*-infected persons^[86,87]. Studies that identify further bacterial as well as host genetic factors that place the patient at greatest risk of disease progression may enhance our approach to better screening strategies, and will improve the control of *H pylori* infection in affected subjects.

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Management and treatment of lactose malabsorption

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Abstract

Lactose malabsorption is a very common condition characterized by intestinal lactase deficiency. Primary lactose malabsorption is an inherited deficit present in the majority of the world's population, while secondary hypolactasia can be the consequence of an intestinal disease. The presence of malabsorbed lactose in the colonic lumen causes gastrointestinal symptoms. The condition is known as lactose intolerance. In patients with lactase nonpersistence, treatment should be considered exclusively if intolerance symptoms are present. In the absence of guidelines, the common therapeutic approach tends to exclude milk and dairy products from the diet. However, this strategy may have serious nutritional disadvantages. Several studies have been carried out to find alternative approaches, such as exogenous β -galactosidase, yogurt and probiotics for their bacterial lactase activity, pharmacological and non pharmacological strategies that can prolong contact time between enzyme and substrate delaying gastrointestinal transit time, and chronic lactose ingestion to enhance colonic adaptation. In this review the usefulness of these approaches is discussed and a therapeutic management with a flow chart is proposed.

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Key words: Lactose; Malabsorption; Intolerance; Therapy

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INTRODUCTION

Lactose malabsorption is a very common condition

characterized by lactase deficiency, an enzyme occurring in the brush border membrane of the intestinal mucosa that hydrolyzes lactose to its components galactose and glucose^[1]. High concentrations of this enzyme are physiologically present in neonates. Post weaning, a genetically programmed and irreversible reduction of its activity occurs in the majority of the world's population^[2] which results in primary lactose malabsorption. Secondary hypolactasia can be the consequence of any condition that damages the small intestinal mucosa brush border or significantly increases the gastrointestinal transit time. Thus, secondary hypolactasia is transient and reversible^[3].

The presence of malabsorbed lactose in the colonic lumen does not necessarily result in gastrointestinal symptoms. Only when lactose malabsorption is associated with clinical manifestations as bloating, flatulence, abdominal pain and diarrhea, "lactose intolerance" occurs^[4].

Diagnosis of lactose intolerance is often made on a clinical basis and in response to an empirical trial of dietary lactose avoidance. A number of methods are available to diagnose lactose malabsorption^[4-8]. The lactose breath hydrogen test is nowadays considered a very simple and useful test in subjects with suspected lactose malabsorption. Undigested lactose is fermented by the colonic microflora with production of hydrogen detectable in pulmonary excretion^[6]. Direct lactase enzyme activity performed on small intestinal tissue biopsy samples may be utilized, but it is an invasive procedure and its reliability can be low because disaccharidase activity in a small biopsy specimen does not necessarily reflect the jejunal activity as a whole^[4]. Recent evidence suggests that a genetic test of the -13910 C/T polymorphism can be used as the first stage screening test for adult-type hypolactasia^[9,10].

THERAPEUTIC STRATEGIES

In patients with lactase nonpersistence, treatment is considered exclusively in the presence of intolerance symptoms^[11]. In the absence of guidelines, the common therapeutic approach tends to exclude milk and dairy products from the diet. However, this strategy may have serious nutritional disadvantages, chiefly for reduced intake of substances such as calcium, phosphorus and vitamins, and may be associated with decreased bone mineral density^[12,13]. To overcome these limits, several studies have been carried out to find alternative approaches, such as exogenous β -galactosidase, yogurt and probiotics for their bacterial lactase activity, pharmacological and non

pharmacological strategies that can prolong contact time between enzyme and substrate delaying gastrointestinal transit time, and chronic lactose ingestion to enhance colonic adaptation.

Exogenous β -galactosidase

Enzyme-replacement therapy with microbial exogenous lactase (obtained from yeasts or fungi) represents a possible strategy for primary lactase deficiency. Enzymes can be added in a liquid form to milk before its consumption or administered in a solid form (capsules or tablets) together with milk and dairy products. Several studies were conducted adding the soluble enzyme to milk some hours before its consumption, thus obtaining a "preincubated milk" [9, 14-17]. This strategy is effective in reducing both H₂ breath excretion and subjective manifestations of discomfort after milk ingestion. However, these trials were carried out on relatively small series populations. They were not placebo-controlled, and results were not comparable since there was a lack of homogeneity in patient subsets. Furthermore, preincubated milk was not considered practical because of the necessity to add the enzyme some hours before its consumption. The low-lactose milk is a preincubated milk in which the lactose is already prehydrolyzed; this product is commercially available but not distributed everywhere (i.e. restaurant, cafeterias, etc). To obviate these problems, several studies have been carried out to show the effectiveness of replacement therapy even when lactase is administered at mealtime [18-22].

In a recent double-blind, placebo-controlled crossover study in 30 intolerant subjects with lactose malabsorption, we showed that both "preincubated milk" and milk treated at mealtime are similarly able to significantly reduce H₂ excretion and symptom score, suggesting that enzymes can be used at mealtime because of its better practicality [23].

While the efficacy of liquid exogenous lactase in reducing both H₂ excretion and symptoms is widely emphasized, results about the exact rate of efficacy are somehow discordant. Several factors may justify these discrepancies. The different enzyme origin can play a role. It is well-known that, at the same dose, enzymes obtained from different microorganisms have different efficacy in hydrolyzing lactose. In fact, comparative studies have already shown the higher efficacy of lactase derived from *K. lactis* than that of the enzyme obtained from *A. niger* [5, 19]. The contribution of residual intestinal mucosal lactase activity should also be considered to explain the variation in intolerance symptoms experienced by lactose malabsorbers. The results of non crossover studies without considering intra-individual variables can be biased, if the residual intestinal mucosal enzyme activity is ignored. Moreover, the different dosage of the enzyme could be another influencing factor. In fact, it is known that a close relationship exists between the amount of lactose to be hydrolyzed and the enzyme units required [5, 21]. Moreover, the stomach pH and bile salt concentrations could influence the efficacy of exogenous lactase.

Solid lactase preparations, in capsules and tablets, are commercially available alternatives for enzyme-replacement therapy. Several studies have investigated and confirmed their efficacy [25-26]. However, comparative studies have

shown that these preparations are more expensive and significantly less effective than prehydrolyzed milk probably due to the enzyme gastric inactivation [11, 17]. Their use can be suggested for solid dairy products.

Therapy compliance with β -galactosidase is assured by good palatability [16, 21, 23] though there are some reported taste alterations [27]. The safety of lactase preparations has recently been confirmed [28]. In conclusion, the addition of exogenous lactase, especially at mealtime, seems to be effective, practical and with no side effects.

Yogurt and probiotics

It is well-known that fermented milk products improve lactose digestion and symptoms of intolerance in lactose maldigesters [17, 29, 30]. Onwulata *et al* [17] demonstrated that commercially available plain yogurt is as effective in reducing H₂ and symptoms as prehydrolyzed milk. The use of fermented milk is based on the presence of endogenous lactase activity of yogurt microorganisms. Yogurt is made of milk incubated mainly with two species of lactic acid bacteria, *L. bulgaricus* and *S. thermophilus* [29, 31]. These microorganisms participate in lactose hydrolysis both during fermentation processes and after lactose ingestion [32-34]. It has been calculated that fermentation decreases lactose content by approximately 25-50% [33-34]. At the same time, this process results in an acid taste and just for this tartness several subjects dislike yogurt. To overcome this limit, the addition of high concentrations of viable *L. acidophilus* to cold milk has been proposed as an alternative to yogurt, obtaining an unfermented milk (sweet acidophilus milk). However, in a comparative crossover study carried out in eleven lactose malabsorbers, Payne *et al* [5] first demonstrated that sweet acidophilus milk does not reduce H₂ excretion and symptoms significantly as milk treated with a commercial lactase preparation. Further studies have confirmed the inadequate effectiveness of sweet acidophilus milk [17, 33, 35]. To explain these findings, it has been proposed that bacterial lactase in sweet acidophilus milk is insufficient to show a measurable effect or is not easily available in the intestinal lumen, becoming accessible only if disruption of the cell membrane occurs [30, 33]. In fact, the cell membrane structures of lactic acid bacteria play a key role in the availability of β -galactosidase [17, 36]. Lin *et al* [37] compared *L. bulgaricus* and *L. acidophilus*, two thermophilic bacteria with similar β -galactosidase activity, bile sensitivity and active transport system for lactose, while differing in the resistance of the cell wall membrane structures, as shown by sonication time for maximum β -galactosidase activity measurements. By measuring H₂ excretion and clinical score, they found that *L. bulgaricus* is a better choice for manufacturing non fermented milk products, because the cell wall membrane structures of *L. bulgaricus* are less tough than those of *L. acidophilus*, with a consequent better ability to release the enzyme.

To effectively release β -galactosidase, bacteria need an intact cell wall as mechanical protection of the enzyme during gastric passage and against the action of bile [38]. It was demonstrated that gastric acid degrades bacterial lactase activity in 20-60 min [39]. However, the association of *L. acidophilus* BG2F04 with omeprazole does not result in reduced hydrogen production and gastrointestinal symp-

toms are not improved after lactose ingestion with respect to lactobacilli without it^[40]. These results could have been due to the selected lactic bacteria. In fact, it is well-known that lactobacilli behave differently depending on the species^[41, 42]. As recently demonstrated, administration of the multiprobiotic product VSL3 cannot reduce H₂ excretion and clinical score^[43]. Further investigations are necessary to clarify the probiotics role in lactose intolerance therapy, also considering their well-known beneficial effects on intestinal functions, gas metabolism and motility^[38].

The bacterial β -galactosidase activity of yogurt is considered to be the main factor responsible for improving lactose digestion; its greater osmolality and energy density can also play a role^[38]. Yogurt delays gastric emptying and intestinal transit causing slower delivery of lactose to the intestine, thus optimizing the action of residual β -galactosidase in the small bowel and decreasing the osmotic load of lactose^[3, 38].

Strategies influencing gastrointestinal transit

Leichter *et al*^[44] showed that full-fat milk (high energy milk) increase lactose tolerance compared to skimmed milk and aqueous lactose solution. It has been reported that fat improves carbohydrate absorption slowing down gastric emptying and intestinal transit time with a consequent prolongation of contact time between enzyme and substrate^[45-47]. Other studies have not confirmed these results^[48, 49]. In particular, Vesa *et al*^[49] have proved that the ingestion of high energy milk delays more significantly gastric emptying than half-skimmed milk but it does not result in a significant improvement in lactose tolerance. To improve lactose digestion by delaying gastric emptying, co-ingestion of food together with dairy products has also been proposed, and it has been demonstrated that lactose is better tolerated when taken with other foods^[19, 50].

Pharmacological approaches that can modify gastric emptying and intestinal transit have also been considered. A double-blind randomized cross-over placebo-controlled study^[51] evaluated the effect of propantheline and metoclopramide on lactose digestion, and found that propantheline-induced prolongation of gastric emptying improves lactose tolerance as measured by reduced symptoms and H₂ breath concentration compared to placebo or metoclopramide. Loperamide has been proposed for its activity on oral-cecal transit time. Szilagy *et al*^[52] demonstrated that loperamide improves H₂ excretion and symptoms when administered together with milk. Later on, the authors concluded that the use of loperamide is not advisable because of its side-effects and high cost^[53].

Adaptation

Lactase is a non inducible enzyme^[1], but it was also reported that continuous lactose consumption decreases hydrogen excretion and the severity of gastrointestinal symptoms^[54-58]. Decreased hydrogen excretion is not necessarily the consequence of increased lactose digestion but can depend on adaptive phenomena. This "adaptation" is associated with changes in gut microflora as well as in some colonic functions and features. The increased microbial β -galactosidase activity is one of the hypothesized mechanisms. Hertzler *et al*^[59] showed that fecal β -galactosidase activity is increased after daily milk feeding for 10 d. However, the lower H₂ production

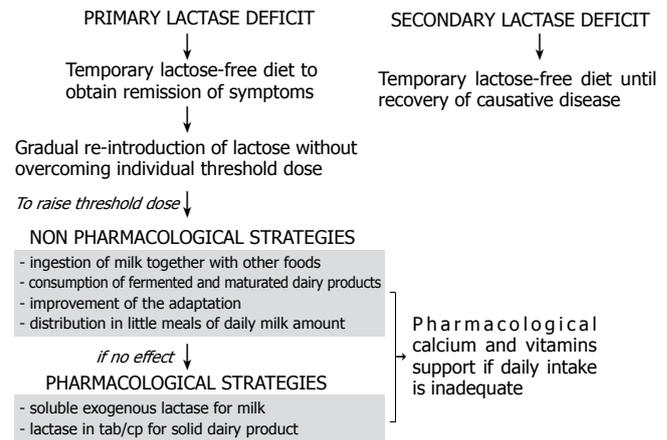


Figure 1 Proposal of therapeutic management in lactose intolerant patients with lactase deficit

is not related to higher levels of lactase in the existing flora. It has been suggested that changes in the intestinal microflora could decrease hydrogen production and/or increase intestinal gas consumption^[60]. Hill *et al*^[61] have proved that malabsorbed lactose enhances the fermentation ability of bifidobacteria and other lactic acid bacteria which can metabolize lactose without hydrogen production. Perman *et al*^[62] hypothesized that the reduction of colonic pH due to the fermentation of malabsorbed lactose affects bacterial metabolism and inhibits hydrogen production. The placebo effect has been suggested as an additional factor to explain the adaptation in response to continuous lactose ingestion. In a controlled double-blind study, Briet *et al*^[59] have demonstrated increased fecal β -galactosidase, reduced H₂ excretion and improved symptoms after lactose ingestion for 13 d. However, in the control group with sucrose, no sign of metabolic adaptation could be found except for a reduced clinical score, suggesting a placebo effect.

PROPOSAL OF THERAPEUTIC MANAGEMENT

We suggest a flow chart for the therapeutic management of lactose malabsorption considering data gathered from literature and our personal experience (Figure 1). We underline that not all subjects with lactase deficit have to be treated, but just symptomatic ones, since there are no known adverse effects of lactose maldigestion other than acute gastrointestinal symptoms^[11]. Furthermore, most lactose-intolerant people can ingest 12 g/d of lactose (equivalent to one cup of milk), without experiencing adverse symptoms^[63-64].

It is necessary to distinguish between primary and secondary lactase deficit. In the secondary form a temporary lactose-free diet is necessary only until a complete recovery of the causative pathological condition is obtained. Lactose breath test can be advised to verify the recovered enzymatic activity. In primary hypolactasia, a different therapeutic strategy should be considered because of the irreversibility of the condition. Initially, a temporary avoidance of milk and dairy products from the diet should be indicated to obtain symptom remission. Subsequently, we suggest a gradual re-introduction of dairy products considering the individual threshold dose, to assure an ad-

equate intake of essential nutritional substances. In order to raise the threshold dose, some non-pharmacological and pharmacological strategies should be considered. Changes in dietary habits, as the ingestion of milk together with other foods such as brioches, cookies and cake, and the consumption of fermented and matured dairy products can assure an adequate milk intake while preventing the onset of intolerance symptoms. Chronic consumption of milk seems to be useful to favorite the adaptation. Considering the dose-dependent lactose absorption, we suggest the distribution of the daily milk amount in small meals. If these strategies fail to reduce lactose intolerance, some pharmacological therapies are available. The addition of exogenous lactase in a liquid form to milk at mealtime has been demonstrated effective and practical. Pre-hydrolyzed milk preparations (i.e. on sale low-lactose content milk) could also be advisable. β -galactosidase tablets or capsules are suitable only for consumption of solid dairy products. Based on the literature data no other pharmacological strategies can be suggested. A pharmacological support of calcium and vitamins is required independently of the chosen therapeutic approach if the daily intake of dairy products is not assured.

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REVIEW

Alterations of tumor suppressor and tumor-related genes in the development and progression of gastric cancer

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Methylation

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Abstract

The development and progression of gastric cancer involves a number of genetic and epigenetic alterations of tumor suppressor and tumor-related genes. The majority of differentiated carcinomas arise from intestinal metaplastic mucosa and exhibit structurally altered tumor suppressor genes, typified by *p53*, which is inactivated via the classic two-hit mechanism, i.e. loss of heterozygosity (LOH) and mutation of the remaining allele. LOH at certain chromosomal loci accumulates during tumor progression. Approximately 20% of differentiated carcinomas show evidence of mutator pathway tumorigenesis due to *hMLH1* inactivation via hypermethylation of promoter CpG islands, and exhibit high-frequency microsatellite instability. In contrast, undifferentiated carcinomas rarely exhibit structurally altered tumor suppressor genes. For instance, while methylation of *E-cadherin* is often observed in undifferentiated carcinomas, mutation of this gene is generally associated with the progression from differentiated to undifferentiated carcinomas. Hypermethylation of tumor suppressor and tumor-related genes, including *APC*, *CHFR*, *DAP-kinase*, *DCC*, *E-cadherin*, *GSTP1*, *hMLH1*, *p16*, *PTEN*, *RASSF1A*, *RUNX3*, and *TSLC1*, can be detected in both differentiated and undifferentiated carcinomas at varying frequencies. However, the significance of the hypermethylation varies according to the analyzed genomic region, and hypermethylation of these genes can also be present in non-neoplastic gastric epithelia. Promoter demethylation of specific genes, such as *MAGE* and *synuclein γ* , can occur during the progressive stages of both histological types, and is associated with patient prognosis. Thus, while the molecular pathways of gastric carcinogenesis are dependent on histological background, specific genetic alterations can still be used for risk assessment, diagnosis, and prognosis.

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Key words: Gastric cancer; *p53*; *E-cadherin*; *hMLH1*;

INTRODUCTION

Ever since the initial report of frequent mutation of the *p53* tumor suppressor gene in primary gastric cancers^[1], a growing number of genetic and epigenetic alterations in tumor suppressor and tumor-related genes have been determined to be involved in gastric carcinogenesis. In addition to *p53* mutations, *epithelial (E)-cadherin* mutations are also frequent and appear to be important in gastric carcinogenesis^[2,3], such that germline mutations of *p53* and *E-cadherin* have both been associated with hereditary gastric cancer^[4,5]. These genes are frequently inactivated by the combination of mutation and loss of heterozygosity (LOH)^[1,3], although *E-cadherin* can also be inactivated by promoter hypermethylation^[6]. LOH at other chromosomal loci can accumulate during gastric cancer progression^[7]. In contrast, mutations of the DNA mismatch repair genes *hMSH2* and *hMLH1* are rare, despite the finding of high-frequency microsatellite instability (MSI) in gastric cancers^[8-10]. Recently, hypermethylation of the *hMLH1* promoter CpG island was found to be responsible for the development of the majority of gastric cancers exhibiting MSI^[11]. Thus, inactivation of *hMLH1* via promoter methylation leads to MSI, and subsequently to mutations in simple repetitive sequences contained within a number of target genes associated with cell proliferation, apoptosis, or mismatch repair, e.g., *transforming growth factor- β type II receptor (TGF- β RII)*, *bcl-2-associated X (BAX)*, *hMSH3*, and *E2F4*^[12]. In addition to *hMLH1*, promoter hypermethylation of tumor suppressor and tumor-related genes such as *APC*, *CHFR*, *COX2*, *DAP-kinase*, *DCC*, *E-cadherin*, *GSTP1*, *HRK*, *LOX*, *MGMT*, *p14*, *p15*, *p16*, *PTEN*, *RASSF1A*, *RUNX3*, *14-3-3 sigma*, *THBS1*, *TIMP-3*, and *TSLC1* has also been described in gastric cancer^[6,11,13-36]. Hypermethylation can occur in both neoplastic and non-neoplastic gastric epithelia, and therefore is regarded as an early event in gastric carcinogenesis. Another epigenetic alteration, hypomethylation, also appears to be involved in gastric carcinogenesis. Global DNA hypomethylation is thought

to occur during the early stages of tumor development in gastric and other tissues, similar to promoter hypermethylation described above^[37-40]. However, demethylation of individual genes, such as *MAGE* and *synuclein-γ*, probably occurs during the progressive stages of gastric carcinogenesis, after global DNA hypomethylation^[41,42].

From a histopathologic point of view, gastric cancers are classified as either differentiated carcinomas, which form tubular or papillary structures (roughly corresponding to the intestinal type), or undifferentiated carcinomas in which such structures are inconspicuous (roughly corresponding to the diffuse type)^[43,44]. It was thought that differentiated carcinomas, with a predominantly intestinal cellular phenotype, originated from gastric epithelial cells that had undergone intestinal metaplasia, while undifferentiated carcinomas rose from native gastric epithelial cells^[43-45]. However, recent advances in mucin histochemistry and immunohistochemistry indicate that some differentiated carcinomas have a predominantly (and, on occasion, exclusively) gastric cellular phenotype and appear to be derived from foveolar epithelial cells^[46,47]. It also appears that gastric cancers can undergo changes in cellular phenotype over time, from gastric to intestinal^[48]. Thus, differentiated carcinomas may develop from native gastric mucosa or intestinal metaplastic mucosa. Therefore, although it has been proposed that different genetic pathways exist for differentiated and undifferentiated histological types^[49], the tumor types must share some common genetic alterations as a significant proportion of differentiated carcinomas progress to become undifferentiated carcinomas^[50]. Indeed, recent studies have indicated that tumor cell phenotype is a marker of particular genetic aberrations^[46,47].

In this article, genetic and epigenetic alterations involved in the development and progression of gastric cancer are reviewed in relation to tumor histogenesis.

GENETIC AND EPIGENETIC ALTERATIONS IN GASTRIC CANCER

p53

The *p53* gene product functions as a cellular gatekeeper and plays important roles in cell growth and division. It assists DNA repair by effecting G₁ arrest in the presence of DNA damage, induces DNA repair genes, and initiates apoptosis if DNA strand breaks fail to repair^[51]. Mutation of *p53* is one of the most prevalent genetic alterations in human cancer, including gastric carcinoma. The gene is usually inactivated through the classic two-hit mechanism, i.e. LOH and mutation of the remaining allele, rather than by DNA methylation^[52]. The frequency of *p53* mutations in early and advanced differentiated gastric carcinomas is consistent at around 40% each, similar to that observed for advanced undifferentiated carcinomas^[53,54]. However, *p53* mutations are rare in early undifferentiated carcinomas^[17,55]. Thus, *p53* gene mutation is thought to be an early event, critical in the development of differentiated carcinomas, and the frequent detection of *p53* mutations in advanced undifferentiated carcinomas is postulated to be due to the frequent conversion of differentiated cancers to an

undifferentiated phenotype as the tumors progress^[50].

hMLH1

Epigenetic methylation-associated inactivation of the *hMLH1* mismatch repair gene is a potent trigger of MSI, especially high-frequency MSI (MSI-H)^[56]. Since the first report of *hMLH1* inactivation associated with DNA methylation in colorectal cancer^[56], similar epigenetic alterations have been described in gastric cancer^[11,13,16]. DNA methylation of *hMLH1* promoter region CpG island is tightly associated with the loss of *hMLH1* expression in gastric cancers exhibiting MSI^[11,13,16]. About 20% of early differentiated carcinomas exhibit MSI-H^[47], while early undifferentiated carcinomas show no evidence of MSI (as described below)^[17]. *hMLH1* methylation is frequently observed in gastric cancers from elderly patients^[51] and has also been described in non-neoplastic gastric epithelia surrounding gastric cancers with MSI^[16,57]. Thus, this field defect may increase the risk of subsequent neoplasia as MSI-H has also been observed in patients with multiple gastric cancers^[58].

E-cadherin

E-cadherin is a member of a family of transmembrane glycoproteins involved in calcium-dependent cell-to-cell adhesion and appears to play a role in organogenesis and morphogenesis^[59]. Germline *E-cadherin* mutations have been reported in familial diffuse-type of gastric cancers^[5]. *E-cadherin* is frequently inactivated via the classic two-hit mechanism in sporadic forms of undifferentiated-scattered (diffuse) type gastric carcinomas, but not in differentiated or undifferentiated adherent type gastric carcinomas^[2,3]. While nearly half of the undifferentiated-scattered (diffuse) type gastric carcinomas contain *E-cadherin* mutations^[2,3], such mutations are rare in early undifferentiated carcinomas^[3,60], and are only detected in the undifferentiated component of mixed differentiated/undifferentiated carcinomas^[61]. This suggests that *E-cadherin* mutations are involved in the de-differentiation of such tumors. In contrast, *E-cadherin* methylation, which is associated with decreased *E-cadherin* expression, is observed in >50% of early stage undifferentiated carcinomas^[6,17], and is also observed in surrounding non-cancerous gastric epithelia^[14,31]. Thus, the epigenetic inactivation of *E-cadherin* via promoter methylation may play a major role in the development of purely undifferentiated carcinomas of the stomach, while mutation of the gene may lead to the de-differentiation of differentiated gastric tumors.

Other tumor suppressor genes

APC gene mutation is a critical genetic event in both the familial and sporadic forms of colorectal tumorigenesis^[62,63]. *APC* mutations are rare in extracolonic cancers, including gastric carcinomas, with less than 10% of both differentiated and undifferentiated gastric carcinomas containing such mutations^[17,46,54,64]. While *APC* promoter methylation has also been reported in colorectal and other human neoplasms^[65], *APC* methylation (promoter 1A) does not appear to be oncogenic in gastric cancer^[18]. Mutation and promoter methylation of *DCC*,

p16, and *PTEN* genes have also been investigated in gastric cancer^[14,23,24,66]. Although few mutations in these genes have been found, the promoter regions of *DCC* and *p16*, but not *PTEN*, exhibit frequent methylation, suggesting that epigenetic inactivation of *DCC* and *p16* may be involved in gastric carcinogenesis^[14,23]. *DAP-kinase* promoter methylation is more frequent in undifferentiated than in differentiated type tumors^[21,30,32]. While *RASSF1A* gene mutations are uncommon, silencing of the gene by promoter methylation is frequent in carcinomas, including gastric carcinomas^[19,67]. *RUNX3*, one of the three mammalian runt-related genes, was recently identified as a tumor suppressor gene that frequently shows loss of expression due to hemizygous deletion and hypermethylation in gastric cancer^[27]. *RUNX3* methylation is mostly cancer-specific, and is observed in about half of all gastric cancer cases^[32]. *TSLC1* has been shown to be inactivated by biallelic methylation in a proportion of primary gastric cancers^[25]. *CHFR* hypermethylation is found to occur concurrently with *hMLH1* hypermethylation and is more frequent in patients over 70 years of age^[68].

Thus, many tumor suppressor and tumor-related genes are methylated in neoplastic and non-neoplastic gastric epithelia, although the significance of hypermethylation is dependent on the analyzed genomic region^[69]. In non-neoplastic gastric epithelia, hypermethylation tends to initially occur in the 5'- and 3'-flanking regions of CpG islands and then spreads toward the transcription start site, whereupon protein expression is shut down. This ultimately results in a field defect that places the affected tissue at an increased risk of gastric cancer development^[29]. Hypermethylation near a transcription start site, which can be cancer-specific and result in gene silencing, can be used as a diagnostic marker of malignancy in tissues or other samples, such as serum or ascites. In addition, hypermethylation at a region next to such a critical region might indicate an early signal of carcinogenesis.

LOH

In differentiated carcinomas of the stomach, frequent LOH has been reported for several chromosomal arms, including 2q, 4p, 5q, 6p, 7q, 11q, 14q, 17p, 18q and 21q^[7,70-73]. However, few reports have focused on the occurrence of LOH in undifferentiated carcinomas, probably due to the difficulty in performing LOH analysis on tissue samples with low tumor cellularity. Nonetheless, frequent LOH at 5q has been reported for both differentiated and undifferentiated tumor types at advanced stages^[74,75]. Apart from a few exceptions, such as the *p53* gene on 17p, the target suppressor gene(s) in the LOH regions on these chromosomal arms remain(s) largely unknown. For example, *IRF-1* on 5q31.1 and *DPC4* (*Smad4*) on 18q21.1 are both located at commonly deleted regions identified in gastric cancer, but exhibit infrequent mutations in gastric cancer^[70,75,76]. The methylation status of the *IRF-1* and *DPC4* (*Smad4*) gene promoter regions remains to be investigated.

MSI

MSI is defined as the presence of replication errors in

simple repetitive microsatellite sequences due to defective DNA mismatch repair, and can be classified as either high-frequency (MSI-H), low-frequency (MSI-L) or stable (MSS)^[77]. The prevalence of MSI in gastric cancer varies among different studies. While some reports suggest that differentiated carcinomas exhibit more frequent MSI than undifferentiated carcinomas^[78], other reports observe the opposite findings^[79]. Again, these contradictory observations may be due to the frequent conversion of differentiated- to undifferentiated-type tumors^[50], as described for *p53* mutations. In a study where MSI analysis was restricted to early differentiated carcinomas (ordinary type), about 20% of tumors were classified as MSI-H^[47]. In contrast, no evidence of MSI has been found in early undifferentiated carcinomas^[17]. Gastric cancers with an MSI phenotype rarely exhibit structural alterations, such as mutations or LOH of tumor suppressor genes^[46,47,80], which suggests that the mutator and suppressor pathways are independent of each other at least in the early stages of gastric carcinogenesis.

Promoter demethylation of *MAGE* and *synuclein-γ*

Melanoma antigen (*MAGE*)-encoding genes are expressed in various tumor types via demethylation of their promoter CpG islands, which are silent in all non-neoplastic tissues except for the testis and placenta. While the function of the *MAGE* peptides is not known, their tumor-specific expression is clearly of great significance to immunotherapy^[81-83]. Demethylation of both the *MAGE-A1* and *-A3* promoters is more frequently observed in gastric cancer patients with advanced clinical stages. These patients also exhibit a higher incidence of lymph node metastasis compared to patients without demethylation^[41]. Furthermore, patients exhibiting *MAGE-A1* and *-A3* promoter demethylation tend to have a worse prognosis, as assessed by the log rank test^[41]. Demethylation of *MAGE-A1* and *-A3* tends to occur during the progressive stages of gastric cancer, and may therefore act as a prognostic factor for gastric cancer patients.

The *synuclein-γ* (*SNCG*) gene, also known as *breast cancer specific gene 1* (*BCSG1*), is a member of the synuclein neuronal protein family, along with *synuclein-a* (*SNCA*) and *synuclein-β* (*SNCB*)^[84-86]. *SNCG* protein expression is highly tissue-specific, being expressed at presynaptic terminals in the brain and peripheral nervous system^[85,86]. However, this tissue specificity is lost during breast and ovarian cancer disease progression^[87]. While *SNCG* expression is normally silent in the breast and ovary, it becomes abundantly expressed in the vast majority of advanced-stage breast and ovarian cancers^[87]. *SNCG* demethylation is also found to be more frequent in primary gastric cancers positive for lymph node metastasis than in metastasis-negative cancers, and more frequent in stage II-IV cancers than in stage I cancers^[42]. An increased tendency for gastric cancer patients with poor prognoses to show *SNCG* demethylation compared to gastric cancer patients with normal methylation has also been reported^[42].

Global DNA hypomethylation is thought to occur during the early stages of tumor development in gastric and other tissues^[37-40]. However, *MAGE-A1*

and *-A3* demethylation are very rare in various organs obtained at autopsy from various age groups^[41], and only partial demethylation of *SNCG* is present in non-neoplastic gastric epithelia^[42]. Therefore, we hypothesize that demethylation of these genes occurs during the progressive stages of gastric carcinogenesis, after global DNA hypomethylation.

GENETIC AND EPIGENETIC ALTERATIONS IN PRECANCEROUS LESIONS

Gastric adenoma/dysplasia

The histopathologic criteria for the diagnosis of gastric intramucosal neoplasia are not universal, and differences in the diagnostic criteria used by Japanese and Western pathologists have been recognized^[88]. It is reasonable to suggest that the discrepant results obtained from the genetic analyses of lesions may be explained by the differences in histopathologic criteria, although a worldwide accepted histological classification has recently been proposed^[89,90]. In my experience, gastric adenomas rarely exhibit genetic alterations, such as *p53* mutation, LOH, or MSI^[10,13,34], with mutations of *APC* gene being the only relatively frequent (20%) DNA structural alteration^[91]. Indeed, *APC* gene mutations are more frequent in gastric adenomas than in differentiated or undifferentiated gastric carcinomas^[17,46,54,64]. More recently, we reported that results of the Padova international classification^[89] correlated with both molecular and cellular phenotypic profiles, and that *p53* and hMLH1 immunohistochemistry clearly discriminated these lesions^[92]. Histopathologic observations have suggested that malignant transformation of gastric adenomas is infrequent, occurring in only 2.5% of conventional protruded and 5.0% of depressed adenomas^[93]. However, detection of certain genetic alterations, such as *p53* mutations, LOH, or MSI, in adenomas may be indicative of malignant transformation^[94]. It is noteworthy that gastric-type intramucosal neoplasia, often diagnosed as adenoma or dysplasia^[95], frequently shows a mutator defect^[16].

Gastric intestinal metaplasia/non-neoplastic gastric epithelia

Intestinal metaplasia may be a precursor of differentiated carcinomas. This concept is supported by the finding that *p53* mutations are detected in gastric intestinal metaplasia, especially incomplete-type, in patients with gastric cancer^[96]. Although frequent MSI has been reported in intestinal metaplasia^[97], there is little evidence of mismatch repair defects in this tissue^[98]. *Helicobacter pylori* infection can accelerate the hypermethylation of genes such as *E-cadherin*^[99]. Hypermethylation of tumor suppressor and tumor-related genes increases with age, and is thought to result in field defects in different organs^[100], although the significance of the hypermethylation appears to be dependent on the genomic region analyzed^[69], as described above for gastric cancer.

CONCLUSIONS

The molecular pathways of gastric carcinogenesis are

dependent on the histological background, such that different genes are affected in different histologies. DNA structural alterations, including *p53* gene mutation and LOH, occur predominantly within intestinal metaplastic mucosa. Hypermethylation of tumor suppressor and tumor-related genes can occur in both metaplastic and native gastric epithelial cells, although at least some of the genes involved, such as *E-cadherin*, are more prone in the latter. Approximately 20% of differentiated carcinomas display evidence of mutator pathway tumorigenesis due to *hMLH1* hypermethylation. Demethylation of *MAGE* and *synuclein-γ* tends to occur during progressive disease stages. Thus, these genetic and epigenetic alterations can be used in the risk assessment, diagnosis and prognosis of gastric cancer.

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E-cadherin in gastric cancer

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Abstract

Cadherin is an adhesion molecule and a superfamily of calcium-mediated membrane glycoproteins. E-cadherin is the prototype of the class E-cadherin that links to catenins to form the cytoskeleton. Recent evidence has shown that E-cadherin not only acts as an adhesive, but also plays important roles in growth development and carcinogenesis. It has been recently viewed as an invasion as well as a growth suppressor gene. This review summarizes the recent discoveries on E-cadherin and its role in gastric cancer. In particular, our work on E-cadherin in gastric cancer, including its relation with *Helicobacter pylori* and clinical applications, are described in detail.

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INTRODUCTION

Although decreasing in incidence, gastric cancer remains a major medical problem and is the second most common fatal cancer worldwide. In spite of intense interest and extensive investigations, its prognosis has not been improved significantly in recent years. The dramatic variations in the incidence of gastric cancer in different geographic areas and from one generation to the next have led to the hypothesis that the incidence of gastric cancer is determined largely by environmental rather than genetic factors. The identification and the subsequent classification of *Helicobacter pylori* (*H. pylori*) infection as type I carcinogen by the WHO sparked a significant understanding in the

etiology of gastric cancer. The current discovery of germline mutation at E-cadherin gene in familial gastric cancers and the findings of IL-1 polymorphisms with increased gastric cancer risk again turn the focus towards the studying of host genetics. The molecular studies of the host may give a definite proof of the etiological role of *H. pylori* in inducing gastric carcinoma.

Only a small fraction of all cancers arise in individuals who carry a germline defect conferring genetic predisposition. Nevertheless, many genes that underlie inherited cancer syndromes have more widespread roles in sporadic cancers, as a result of somatic mutations that arise during tumor initiation or progression. Hence, in this review paper, we shall focus on the E-cadherin gene, one of the candidate genes in gastric cancer, and the work we have done on E-cadherin in gastric carcinogenesis.

E-CADHERIN

Cadherin is a superfamily of calcium-mediated membrane glycoproteins, with a molecular mass of 120 ku, forming one of the four classes of adhesion molecules^[1-3]. Some common cadherins expressed by epithelial cells are E-cadherin, N-cadherin and P-cadherin. The intracellular domains of classical cadherins interact with β -catenin, γ -catenin (also called plakoglobin) and p120ctn to assemble the cytoplasmic cell adhesion complex (CCC) that is critical for the formation of extracellular cell-cell adhesion. β -catenin and γ -catenin bind directly to α -catenin, which links the CCC to the actin cytoskeleton^[4,5]. The cadherins are responsible for the homotypic cell-cell adhesion. However, knowledge gained in the recent few decades has shown that E-cadherin not only acts as an adhesive, but also plays important roles in growth development and carcinogenesis.

E-CADHERIN AND CANCER

Role of E-cadherin in metastasis

E-cadherin is the prototype of the cadherin class. It is expressed in all epithelial cell types. Underexpression of the E-cadherin is found in gastric and other cancers, and correlates with infiltrative and metastatic ability^[6]. It has been proposed that the loss of E-cadherin-mediated cell-cell adhesion is a prerequisite for tumor cell invasion and metastasis formation^[7]. Re-establishing the functional cadherin complex, e.g. by forced expression of E-cadherin, results in a reversion from an invasive, mesenchymal, to a benign, epithelial phenotype of cultured tumor cells^[7,8]. Hence, the E-cadherin gene is also called as an invasion

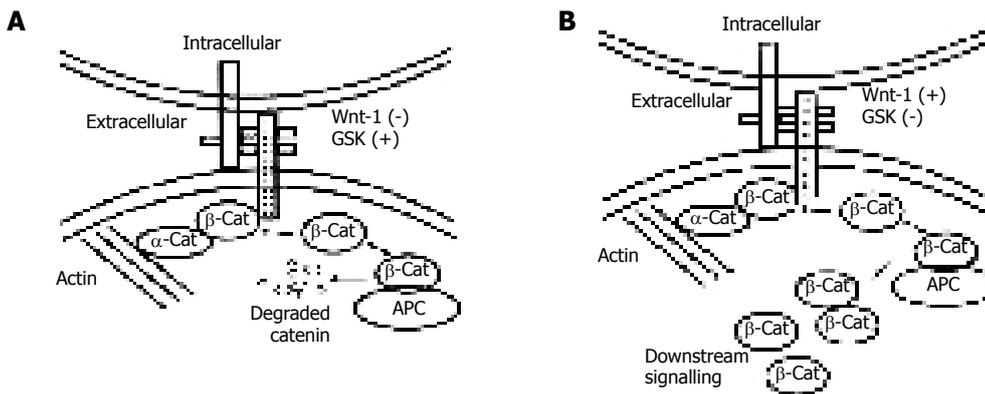


Figure 1 Illustrated interaction between the cadherin-catenin complex and the APC protein. (A) In the absence of Wnt-1 and the presence of glycogen synthase kinase (GSK), β -catenin (β -cat) is stabilized and bound to cadherin or APC protein. Cadherin acts as a negative regulator of β -catenin by regulating the amount of free β -catenin. The free cytoplasmic β -catenin is degraded. (B) In the presence of Wnt-1, GSK is antagonized and mutant APC or tyrosine phosphorylated β -catenin cannot bind to each other, cytosolic free β -catenin concentrations rise, which leads to down-stream cell signalling and may be involved in carcinogenesis.

suppressor gene.

Role of E-cadherin in oncogenesis

Recently, it has been postulated that the role of E-cadherin in carcinogenesis does not limit only to metastasis and invasion (Figure 1). It is now being increasingly recognized that there is also a possible role of E-cadherin in modulating intracellular signaling, and thus promoting tumor growth. There are several lines of evidence. Cadherin-mediated cell-cell adhesion can affect the Wnt-signaling pathway^[9,10]. β -catenin (as well as γ -catenin) is usually sequestered by cadherins in the cadherin-catenin complex. Upon loss of E-cadherin function, non-sequestered, free β -catenin is usually phosphorylated by glycogen synthase kinase 3 β (GSK-3 β) in the adenomatous polyposis coli (APC)-axin-GSK-3 β complex and subsequently degraded by the ubiquitin-proteasome pathway. In many cancer cells, loss of function of the tumor suppressor APC, mutations in β -catenin or inhibition of GSK-3 β by the activated Wnt-signaling pathway leads to the stabilization of β -catenin in the cytoplasm. Subsequently, it translocates to the nucleus, where it binds to the members of the Tcf/Lef-1 family of transcription factors and modulates expression of Tcf/Lef-1-target genes, including the proto-oncogene c-myc and cyclin D1. In addition, recent studies on familial gastric cancer indicate that E-cadherin can also act at a much earlier stage during tumor development. Mutations of the E-cadherin gene were found in three familial gastric cancer kindred from New Zealand^[11] and this observation was confirmed in the kindred of European origin^[12]. These results demonstrate that loss of function of E-cadherin may play a role in susceptibility to initial tumor development in addition to its role as an inhibitor of tumor invasion.

EXPRESSION OF E-CADHERIN IN GASTRIC CANCER

The expression of E-cadherin has been studied by immunohistochemical method. Decreased expression has been observed in gastric cancer by various authors,

ranging from 17%^[13] to 92%^[14], depending on the method and the definition used. The decreased expression of E-cadherin was mainly observed in diffuse type and less in intestinal type of gastric cancer. Direct correlation between E-cadherin and the grade of tumor differentiation has been observed in all these studies. In addition, it was shown in a study of 413 gastric cancers by Gabbert *et al.*^[15] that patients with E-cadherin positive tumors had significantly better 3- and 5-year survival rates than patients with E-cadherin negative tumors. A similar trend of decrease in disease-free survival was also observed in other cancers exhibiting downregulation of E-cadherin^[16].

We have also studied the change in the expression of the E-cadherin complex along the Correa's cascade in gastric cancer by immunohistochemical staining^[17]. We observed that the staining pattern, intensity and the percentage of cells with positive stains decreased along the Correa's cascade; that is, the staining was strong and membranous in normal gastric epithelium, but gradually decreased in intensity and percentage, as well as the pattern was changed towards cytoplasm in chronic atrophic gastritis, intestinal metaplasia, dysplasia and eventually to adenocarcinoma.

However, methods to evaluate, qualitatively or quantitatively, protein expression in biopsies from human tumors may have serious limitations because of sampling from heterogeneous tissue, non-stoichiometric labeling and subjective evaluation.

MECHANISM OF INACTIVATION OF E-CADHERIN

Genetic inactivation

The E-cadherin gene can be genetically inactivated by a number of mechanisms. The first hit of a role for E-cadherin in tumor development, particularly in suppression of invasion, came from loss of heterozygosity studies on chromosome 16. Subsequently, mutations were reported in tumor samples in diffuse type gastric carcinomas^[18], gynecologic cancers^[19], and infiltrative lobular breast cancer^[20,21]. In gastric cancer, E-cadherin mutations are common in

diffuse type carcinomas, but not seen in intestinal type^[18]. The specificity of types of cancer affected by mutations of the E-cadherin gene, despite the prevalence of reduced E-cadherin expression in many cancer types, suggests that E-cadherin mutations may be of particular importance in the development of these tumors. In addition, mutations in diffuse gastric cancer^[22] have been detected early in tumor development, suggesting a role in tumor suppression, in addition to invasion suppression. Further evidence for this comes from the observations that mutations of E-cadherin have also been observed in several kindred exhibiting familial gastric cancer^[11,12]. Furthermore, at least one kindred exhibited both diffuse gastric cancer and early-onset breast cancer^[11].

Inactivation by hypermethylation

In recent years, it has become increasingly apparent that increased methylation within the promoter regions of genes plays a key role in the inactivation of many important genes during the development of cancer^[23]. Subsequently, numerous reports of E-cadherin promoter methylation, associated with reduced E-cadherin expression, have been published. Hypermethylation at E-cadherin has also been shown to play a role in familial gastric cancer, acting as the second hit (the first hit is either mutation or loss of heterozygosity) in inactivation of E-cadherin^[24].

We have previously shown that the immunostaining of E-cadherin decreases along the Correa's cascade; therefore, we went on to study the underlying mechanism. We found that the frequency of methylation at E-cadherin also increases along the Correa's cascade, with the highest frequency detected in the tumorous lesions and metastatic lesions^[25]. The methylation frequency correlated with the tumor depth and node invasion. In addition, most of the decrease in immunostaining was accounted for by the presence of methylation at E-cadherin. More importantly, we also observed that *H pylori* infection was also associated with E-cadherin methylation.

In fact, the role of *H pylori* in the regulation of E-cadherin has been studied recently. It has been shown that *H pylori* infection is associated with downregulation of E-cadherin, probably by generating cell signaling events that counteract the normal function of protein kinase C^[26,27]. The resulting increase in permeability mediated by the reduction in cell adhesion might allow *H pylori* antigens to reach the gastric lamina propria and activate the mucosal immune system, with resultant tissue damage.

In relation to the above findings, we therefore hypothesized that by eradicating *H pylori*, methylation at E-cadherin may disappear^[28]. We performed a prospective randomized trial. Patients with dyspepsia and *H pylori* infection were randomized to receive *H pylori* eradication therapy (Group 1, $n = 41$) or no treatment (Group 2, $n = 40$) and were followed up prospectively. Gastric mucosae were taken for methylation assay at week 0 (before treatment) and week 6 (after treatment). Methylation was assessed using methylation-specific PCR. Methylation was detected in 46% (19/41) and 17% (7/41) at week 0 and 6, respectively in Group 1 ($P = 0.004$). 78.9% (15/19) specimens turned unmethylated after eradicating *H pylori*. Methylation was detected

in 47.5% (19/40) and 52.5% (21/40) at weeks 0 and 6, respectively in Group 2 ($P = 0.5$). Archived specimens with intestinal metaplasia with *H pylori* infection ($n = 22$) and without the infection ($n = 19$) were also retrieved for methylation analysis. But methylation frequency did not differ in *H pylori* positive or negative intestinal metaplastic specimens (72.7% vs. 63%, $P = 0.5$). Therefore, we postulated that *H pylori* eradication therapy could reverse methylation in patients with chronic gastritis only and may halt the process of gastric carcinogenesis.

Postulated mechanism of helicobacter pylori infection for inducing methylation

Experimental data from *in vitro* studies support our contention that E-cadherin methylation might be related to *H pylori* infection. El-Omar *et al*^[29] reported that interleukin-1 β polymorphism that led to upregulation of interleukin-1 β with *H pylori* infection was associated with increased risk of gastric cancer. Furthermore, Hmadcha *et al*^[30] found that interleukin-1 β , through the production of nitric oxide and the subsequent activation of DNA methyltransferase, might induce gene methylation. It is thus possible that *H pylori* induces methylation through the production of interleukin-1 β and hence the downstream activation of nitric oxide and DNA methyltransferase, which subsequently led to an increased risk of E-cadherin methylation and hence gastric cancer^[31].

OTHER REGULATORY MECHANISMS OF E-CADHERIN

Loss of E-cadherin function during tumor progression can also be caused by transcriptional repression binding to the CDH1-E box elements, e.g. by the repressors Snail^[32,33] and Sip-1^[34]. Tyrosine phosphorylation has also been previously implicated in the regulation of cadherin function: RTKs, such as EGFR, c-Met and FGFR, and the non-receptor tyrosine kinase, c-Src, phosphorylate E-cadherin, N-cadherin, β -catenin, -catenin and p120ctn, resulting in the disassembly of the cytoplasmic adhesion complex and a disruption of cadherin-mediated cell adhesion and cell scattering^[35-37].

SOLUBLE E-CADHERIN

E-cadherin has a cleavage site near the transmembrane domain and artificially produces a soluble 80 ku amino-terminal fragment in the culture medium upon trypsin digestion in the presence of calcium^[38]. This soluble E-cadherin fragment is considered to be a degradation product of the 120 ku intact E-cadherin generated by a calcium-dependent proteolytic action, and can be detected in the protein extract of tissue samples from peripheral blood and urine in normal subjects^[39]. Serum soluble E-cadherin is reported to be increased in dermatological disorders (bullous pemphigoid, pemphigus vulgaris, psoriasis vulgaris), multi-organ failure, and various tumors like bladder cancer, prostate cancer, lung cancer and gastric cancer^[39-43]. The role of soluble E-cadherin and its biological significance is still, at present, unclear. Recent studies indicate that, in inflammatory condition, serum

soluble E-cadherin is induced by inflammatory mediators and cytokines^[44]; whereas in cancerous diseases, soluble E-cadherin is increased by cleavage of tissue E-cadherin due to overexpressed proteases^[45]. The potential of soluble E-cadherin to be a prognostic marker in cancerous diseases has been shown in bladder cancer^[40] and gastric cancer^[46].

We have examined the potential clinical application of E-cadherin. We first studied the correlation between E-cadherin immunostaining expression and the concentration in sera^[47]. We found that normal strong membranous staining, e.g. in normal gastric epithelium, was associated with low levels of serum soluble E-cadherin, but a partially reduced; cytoplasmic staining, e.g. in intestinal type of gastric cancer, was associated with high levels; whereas complete absence of staining, e.g. in diffuse type of gastric cancer, was associated with low levels of soluble E-cadherin. We also found that soluble E-cadherin may be a potentially useful prognostic marker. High levels of soluble E-cadherin correlated with the depth of tumor invasion, as well as inoperability^[48]. More importantly, levels higher than 10 000 ng/mL predicts survival less than 3 years in more than 90% of patients^[47]. Currently, we are investigating the use of post-operative soluble E-cadherin levels to predict tumor recurrence in patients who have received curative surgery for gastric cancer.

CONCLUSION

Being an adhesion molecule, E-cadherin plays an important role in invasion and metastasis in almost all kinds of epithelial malignancies, and hence is named invasion suppressor gene. However, recent studies have shown that E-cadherin actually plays an early and important role in carcinogenesis and acts as a tumor suppressor gene, particularly in gastric cancer. Further thorough understanding of the role of E-cadherin and its association with extracellular environment and intracellular functions will be extremely important. Potentially, reversing methylation at E-cadherin in the gastric epithelium in patients with *H pylori* infection may halt the process of future development of gastric cancer. In addition, soluble E-cadherin may be a useful prognostic marker for gastric cancer.

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REVIEW

Chemotherapy for gastric cancer

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Abstract

Metastatic gastric cancer remains a non-curative disease. Palliative chemotherapy has been demonstrated to prolong survival without quality of life compromise. Many single-agents and combinations have been confirmed to be active in the treatment of metastatic disease. Objective response rates ranged from 10-30% for single-agent therapy and 30-60% for polychemotherapy. Results of phase II and III studies are reviewed in this paper as well as the potential efficacy of new drugs. For patients with localized disease, the role of adjuvant and neoadjuvant chemotherapy and radiation therapy is discussed. Most studies on adjuvant chemotherapy failed to demonstrate a survival advantage, and therefore, it is not considered as standard treatment in most centres. Adjuvant immunochemotherapy has been developed fundamentally in Korea and Japan. A meta-analysis of phase III trials with OK-432 suggested that immunochemotherapy may improve survival of patients with curatively resected gastric cancer. Based on the results of US Intergroup 0116 study, postoperative chemoradiation has been accepted as standard care in patients with resected gastric cancer in North America. However, the results are somewhat confounded by the fact that patients underwent less than a recommended D1 lymph node dissection and the pattern of recurrence suggested a positive effect derived from local radiotherapy without any effect on micrometastatic disease.

Neoadjuvant chemotherapy or chemoradiation therapy remains experimental, but several phase II studies are showing promising results. Phase III trials are needed.

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TREATMENT OF ADVANCED GASTRIC CANCER

Should chemotherapy be offered to patients with advanced gastric cancer?

Traditionally, although responses to chemotherapy have been reported in up to 60% of patients in phase II trials, most patients developed drug resistance within few months, and median survival of treated patients usually ranged from 7 to 9 mo. These disappointing results together with the toxic effects derived from chemotherapy prompted to several investigators to evaluate the benefit of chemotherapy in terms of survival and/or quality of life compared with best supportive care alone. Four randomized phase III studies were conducted during the last decade, showing a survival advantage in favour of chemotherapy of about 6 mo^[1-4]. In the light of these findings, chemotherapy should be offered to patients with metastatic gastric cancer in good general conditions. Even in those patients with asymptomatic disease, a similar survival advantage derived from early chemotherapeutic treatment has been shown as compared with conservative attitude and therapy only when symptoms appear^[5]. This benefit was obtained without quality of life compromise.

Which drugs can be considered active as single agents?

Many traditional and modern drugs have been tested as single-agent in advanced gastric cancer. Table 1 shows those agents that have obtained $\geq 10\%$ response rate. It is worth to remark that classical drugs were tested in the 1960s and 1970s and its true single-agent activity may be overestimated due to the methodology employed. In that sense, in the setting of a randomized trial reported in 1994, both epirubicin and 5-fluorouracil obtained less than 10% response rate^[6]. Complete responses with single-agent therapy are uncommon, and partial responses ranged 10-20%, followed by a short period of time to disease progression.

Combination therapy for advanced gastric cancer patients

Several combination regimens have been developed with the aim of improving overall response rate and survival. Platinum compounds, 5-fluorouracil, and recently taxanes and irinotecan might be considered the mainstay of these

Table 1 Single agent activity in advanced gastric cancer

| Agents | Response rate (%) |
|------------------|-------------------|
| Mitomycin C | 30 |
| Doxorubicin | 17 |
| Epirubicin | 19 |
| Cisplatin | 19 |
| BCNU | 18 |
| 5-Fluorouracil | 21 |
| Etoposide (oral) | 21 |
| Hydroxurea | 19 |
| UFT | 27 |
| Capecitabina | 19 |
| S-1 | 45 |
| Paclitaxel | 17-23 |
| Docetaxel | 17-29 |
| CPT-11 | 18 |

combinations (Table 2).

Fluorouracil-based combinations: The FAM (fluorouracil-doxorubicin-mitomycin C) combination was widely used in the 1980s. After the first publication where the authors reported an overall response rate of 42%^[7], more than six hundred patients were treated with FAM or modified-FAM schedules reporting a cumulative response rate of 30%^[8]. The concept of biochemical modulation of the activity of 5-FU led several authors to design schedules, such as FAMTX, FEMTX and EFL. In 1982, Klein *et al*^[9] reported a response rate of 63%, administering sequentially high-dose methotrexate, with leucovorin rescue, and 5-FU followed by adriamycin (FAMTX) on day 15, with a median survival of 9 mo. In a subsequent study conducted by the EORTC with the same regimen, 22 out of 67 (33%) patients responded and nine of them achieved clinical complete responses. The median survival for all patients was 6 mo. Toxicity consisted mainly of leukopenia (WHO grade 4 in 14%), mucositis (WHO grade 3 in 11%), and alopecia^[10]. When adriamycin was substituted by its analogue 4-epi-doxorubicin (FEMTX), no advantages were found in response rate or tolerance^[2]. It is well known that 5-fluorouracil modulated by leucovorin combined with intravenous etoposide (EFL) is a well tolerated scheme, initially designed for elderly patients or those with cardiac risk. A phase II trial reported a 53% response rate, including 12% of complete responses^[11]. Overall survival was 11 mo. The results of three modified-EFL regimens consisting of oral etoposide, oral leucovorin and either 5-FU continuous infusion or tegafur were controversial. Colleoni *et al*^[12] and Feliu^[14] reported 42% and 26% of partial remissions, and 8% and 9% complete responses respectively. Median survival times were 9.5 and 9 mo, respectively. Toxicity with these schedules was mild or moderate, mainly nausea and vomiting, and asymptomatic leukopenia. On the other hand, only 16% of patients treated with the entirely oral tegafur-containing schedule designed by Raderer *et al*^[13] achieved partial remission, and overall survival was 6 mo. The investigators argued a low compliance due to severe emesis, in spite of prophylactic intake of 5-HT₃ receptor antagonists, to explain these disappointing results.

Combinations based on cisplatin/5-FU continuous

Table 2 Combination therapy for advanced gastric cancer

| | Response rate (%) | Median survival (mo) |
|----------------------------------|-------------------|----------------------|
| FU-based combinations | | |
| FAM | 30-42 | 6-9 |
| FAMTX | 33-63 | 6 |
| ELF | 53 | 11 |
| Modified-ELF | | |
| Cisplatin-5-FU CI synergism | | |
| PF | 40 | 9-10 |
| ECF | 59-71 | 8.7 |
| P-5-FU 48-h CI | 50 | 9.3 |
| P-5FU 24-h CI | 58 | 11 |
| Cisplatin-based combinations | | |
| EAP | 33-64 | 9 |
| FLEP | 35 | 8 |
| FLEP-type | 39 | 11 |
| LV5FU2-P | 27 | 13.3 |
| Combinations including new drugs | | |
| P-CPT11 | 41-58 | 9-12 |
| LOHP-CPT11 | 50 | 8.5 |
| CPT11-bolus 5-FU | 22 | 7.6 |
| CPT11- 5-FU CI | 20 | 7 |
| P-Xeloda | 54.8 | 10.1 |
| ECC | 59 | 9.6 |
| P-S-1 | 73-74 | 12 |
| TPFU/LV | 50 | 11-14 |
| DP | 37-56 | 9-11 |
| DPF | 51 | 9.3 |

CI: Continuous infusion.

infusion (CI) synergism: The *in vitro* synergy between cisplatin and 5-FU, especially in continuous infusion administration, led several investigators to test the combination of both drugs with or without the addition of a third active agent. About 40% response rate and median survival of 9-10 mo were reported with the combination of cisplatin and 5-FU CI for 5 d^[15,16]. Higher response rates (71% and 59%) were achieved in two phase II trials with the combination of protracted continuous 5-FU infusion, cisplatin and epirubicin (ECF), but apparently with a similar overall survival^[17,18]. The Spanish Group for the Treatment of Digestive Tumors (TTD) conducted a phase II study with cisplatin every 3 wk in combination with a weekly 48-h infusion of 5-FU. Responses were achieved in 50% of patients and median survival reached 9.3 mo^[19]. In these schedules, moderate myelosuppression appeared as the most relevant side effect with few patients requiring hospitalization for neutropenic fever. A multicenter study showed that a weekly 5-FU 24-h continuous infusion modulated by leucovorin and combined with bimonthly cisplatin obtained a 58% response rate and 11 mo of median survival^[20].

Others cisplatin-based combinations: In 1989, a group of German investigators developed a combination regimen consisting of cisplatin + etoposide + doxorubicin (EAP). Responses were observed in 43 of 67 (64%) patients that included 21% clinical complete responses. Median survival time was 9 mo^[21]. With exactly the same schedule employed by Preusser *et al*^[18], 36 previously untreated patients with advanced gastric cancer were

involved in a phase II trial at the Dana-Farber Cancer Institute. Only 33% objective responses, including 8% clinical complete responses were reported. Median survival for the entire group was 8 mo^[22]. Their revision of published experience with EAP-like regimens over 262 patients revealed a mean response rate of 41%. Although EAP is an active regimen, it should be noted the high hematologic toxicity reported with this schedule. Deaths due to treatment-related toxicity have been reported in the majority of series, ranging from 6% to 20%. The FLEP schedule developed by our group combined bolus 5-FU modulated by folinic acid (FA) for three consecutive days with epirubicin and cisplatin on day 2. Objective responses were observed in 32 of 90 patients (35%), including 9% clinical complete remissions, and median survival time was 8 mo. Ten patients presented episodes of febrile neutropenia, but no toxic deaths were observed^[23]. Almost double response rate was reported with an intensive weekly FLEP-modified schedule, but median survival achieved seemed to be slightly better if so^[24]. Other FLEP-type schedule in which cisplatin and 5-FU/FA were combined with etoposide, taking advantage of the three drugs synergism, obtained 39% responses and a median survival of 7 mo^[25]. A well known active regimen in metastatic colorectal cancer (LV5FU2), combined with bimonthly cisplatin infusion obtained one of the higher overall survival of 13.3 mo in patients with advanced gastric cancer^[26]. Hematologic toxicity was the most common adverse event (42.9% grade 3 or 4) but less than 5% of patients experienced febrile neutropenia and no toxic deaths were observed. Nausea and vomiting were also frequent side effects, despite systematic prophylaxis.

Combinations including new active drugs

Irinotecan shows a marked synergism with cisplatin as well as a lack of cross-resistance due to different mechanisms of action and a non-overlapping profile of adverse reactions. Shirao *et al*^[27] reported a 41% response rate with a combination of irinotecan and cisplatin in a phase I-II trial. The recommended dose and schedule were 70 mg/m² of CPT-11 on days 1 and 15 and 80 mg/m² cisplatin on day 1 every 4 wk. The dose-limiting toxicity was neutropenia. A phase II study employing a combination of CPT-11 (70 mg/m²) plus cisplatin (80 mg/m²) in patients with metastatic gastric cancer achieved an overall response rate of 48%^[28]. In Western countries, Ajani *et al*^[29] obtained a very high response rate (58%) with a combination of irinotecan (65 mg/m²) plus cisplatin (30 mg/m²), both administered intravenously 1 d per week for 4 consecutive weeks, followed by a recovery period of two weeks. The median survival reported reached 9 mo. Compared with the classic cisplatin combination regimens, the introduction of CPT-11 as part of the treatment schedule increased the gastrointestinal toxicity. A recent pharmacokinetic study comparing two infusion schedules of irinotecan plus cisplatin suggested some advantages in favour of 24-h continuous infusion of CPT-11. The area under the plasma concentration-time curve of SN-38 was increased by 24-h infusion when compared with the 90-min infusion^[30]. Irinotecan has also been combined with oxaliplatin as first-line treatment for locally advanced or metastatic

gastric cancer patients. Oxaliplatin 85 mg/m² followed by CPT-11 200 mg/m² as a 30-min i.v. infusion achieved an overall response rate of 50% and a median survival of 8.5 mo in a phase II multicentre study conducted in Greece^[31]. A weekly irinotecan plus bolus folinic acid and bolus^[32] or infusion^[33] 5-FU combination did not improve the outcome in patients with advanced gastric cancer in a non-randomised comparison with other CPT-11 combinations. Capecitabine is a novel oral fluoropyrimidine carbamate that mimics continuous infusion of 5-FU. The combination of capecitabine and cisplatin has been proven to be active and well tolerated in patients with advanced gastric cancer, giving an overall response rate of 54.8% in the intention-to-treat analysis^[34]. Evans *et al.*^[35] evaluated in a phase I trial the combination of epirubicin, cisplatin and capecitabine (ECC). With a fixed-dose of cisplatin 60 mg/m² and epirubicin 50 mg/m², both every 3 wk, the recommended dose for capecitabine was 1 000 mg/m² per 12 h on an intermittent schedule (two weeks of treatment, followed by one week rest). An overall response rate of 59% and median survival of 9.6 mo have recently been reported in a phase II trial carried out in Korea^[36]. S-1 is another fluoropyrimidine derivative developed mainly in Japan. A phase I/II study conducted by Koizumi *et al*^[37] found a recommended dose for this combination of cisplatin 60 mg/m² on d 8 plus S-1 40 mg/m² b.i.d for 21 d, followed by a 2-wk rest. The response rate (74%) achieved was the highest published in the literature, and the median survival reached 12 mo. These encouraging results have been confirmed in another phase I/II trial carried out in Japan in which S-1 was administered orally at a dose of 80 mg/m² per d for 2 wk, followed by a 2-wk rest, plus cisplatin 70 mg/m² as the recommended dose. Among eleven evaluable patients for response, 8 achieved a partial response (73%) and 1 had stable disease. The main toxicities were neutropenia, nausea and anorexia, all of them not severe, reversible and manageable^[38]. In Europe, the first phase II trial with S-1 as monotherapy for advanced gastric cancer revealed that the starting dose of 40 mg/m² was not tolerable due to significant diarrhoea. At 35 mg/m² twice daily a response rate of 26.1% was reported according to an independent radiology review^[39]. A recent phase II trial conducted in the United States and Germany was presented at ASCO 2005, employing cisplatin 75 mg/m² on d 1 plus S-1 25 mg/m² p.o twice daily on d 1-21, followed by a 7-d of recovery^[40]. Over 41 assessable patients, the overall confirmed response rate reached 51.2% and the median time to progression was 4.8 mo. The regimen showed a very favourable safety profile, with only 2.1% of patients suffering from febrile neutropenia and less than 15% experienced grade 3 or 4 gastrointestinal toxicities. A phase III trial comparing cisplatin plus S-1 *versus* cisplatin plus continuous infusion 5-FU is ongoing in Western countries. Similar results were reported by Iwase *et al*^[41] in a phase II trial in which S-1 was given orally at 80 mg/m² daily for 14 d and cisplatin was administered as a 24-h continuous infusion on day 8. Many randomized trials consisting of an S-1 based regimen are now being evaluated in Japan. Moreover, 5-FU alone is being compared with the combination of cisplatin plus irinotecan and with S-1 alone in a three-arms controlled study. The accrual of

a study comparing S-1 *versus* S-1 plus cisplatin has recently been completed^[42].

Taxane-containing combinations have considerable activity in the treatment of gastric cancer. Kim *et al*^[43] reported a 51% response rate with the combination of paclitaxel 175 mg/m² by 3-h, cisplatin 20 mg/m² per d for 5 d and 5-FU 750 mg/m² by 24-h continuous infusion for 5 d. In their treated population, there were patients included as second-line chemotherapy. Two consecutive studies from the University of Tuebingen Medical Center in Germany, in chemo-naïve advanced gastric cancer patients, employing weekly 24-h continuous intravenous infusion of 5-FU, modulated by leucovorin, plus 3-weekly cisplatin and either weekly or 3-weekly paclitaxel confirmed an overall response rate of 50% and survival times of 11 and 14 mo, respectively^[44,45]. Docetaxel has also been combined with cisplatin, and in a three-drugs combination together with cisplatin and 5-FU. Two phase II trials combining docetaxel and cisplatin reported response rates of 37-56% and median survival times 9-11.5 mo^[46,47]. Recently, Roth *et al*^[48] have demonstrated that protracted infusion of 5-FU can be safely added to a docetaxel-cisplatin combination, showing a high response rate of 51%. Nevertheless, it should be remarked that although no treatment-related deaths were reported, 79% of patients experienced \geq grade 3 neutropenia and ten febrile neutropenia episodes were recorded. Grade 3 diarrhoea appeared in 19% of patients. The combination of docetaxel plus S-1 has recently been tested in Japan^[49], showing a high activity (52.1% response rate and 93.8% tumour control rate), but probably it would be necessary to add hematopoietic growth factors due to the high rate of grade 4 neutropenia (22.9%).

Which of these combinations could be considered as a standard treatment?

Looking at the data of the phase II trials shown in Table 2, it can be concluded that we have made only marginal progress in the palliative treatment of advanced or metastatic gastric cancer. Despite a relatively high rate of objective responses achieved with cisplatin-based therapy and other combinations, including new agents, the time to treatment progression was usually short and the median survival ranged from 8 to 13 mo. Toxicity profile may be quite different depending on the schedule and it should be taken into account, mainly when great differences in progression-free survival and overall survival are unexpected. Relatively aggressive regimens, such as EAP-like, FLEP or DCF (Docetaxel-cisplatin-5-FU), commonly require regular use of colony-stimulating factors due to high medullar toxicity. ECF or cisplatin plus 5-FU CI seems to be less toxic but the need for central venous access and portable pumps make these schedules less comfortable. New oral fluoropyrimidines might play a role in the future to avoid central venous access.

Nowadays, data derived from randomized phase III studies do not convincingly support the use of a specific schedule as a standard treatment. During the early 1990s, especially in Europe, FAMTX became the gold standard because it was shown to be superior in overall survival when compared with the traditional FAM regimen (42 wk

vs. 29 wk)^[50]. The results of five subsequent randomized trials comparing FAMTX or FAM *versus* cisplatin-based combinations (EAP, EEP, FLEP, and PF) were disappointing^[51-55]. The EORTC trial comparing FAMTX *versus* PF *versus* a non-cisplatin combination (ELF) showed a low activity in the three arms (\leq 20%), no differences in toxicity profile and similar modest survival times below 8 mo^[54]. The authors concluded that none of these schedules should be regarded as standard treatment for advanced gastric cancer and that new strategies should be found. The ECF regimen developed in United Kingdom was compared with FAMTX. Long-term results demonstrated that ECF yielded a significantly higher response rate (46% *vs* 21%, $P=0.00003$), median survival (8.7 mo *vs* 6.1 mo, $P=0.0005$) and two-year survival rates (14% *vs* 5%, $P=0.03$)^[56]. However, the results are somewhat confounded by the fact that around one-third of patients included in both arms had locally advanced disease rather than metastatic disease. More patients in the ECF arm (12 out of 47) underwent surgery compared to the FAMTX arm (5 out of 51), and it is not clear in the study whether there were common criteria to perform a surgical rescue after neoadjuvant chemotherapy, reviewed by a surgical expert panel. On the other hand, ECF resulted in a more favourable toxicity profile than FAMTX. Therefore, many centres in Europe are now considering ECF as their standard practice. A later English study comparing ECF *versus* MCF (mitomicyn C, cisplatin and fluorouracil) over 574 eligible patients showed an equivalent response rate (42.4% for ECF *vs* 44.1% for MCF) as well as median survival times (9.4 mo for ECF *vs* 8.7 mo for MCF), although quality of life assessment suggested that ECF regimen was a more tolerable schedule^[57].

Promising results of phase II studies containing new drugs have prompted to confirm these outcomes in phase III randomized trials. Data of a phase III study comparing DCF (docetaxel-cisplatin-5-FU) *versus* PF (cisplatin-5-FU) reported a significant advantage for DCF in response rate (37% *vs* 25%) and time to progression (5.6 mo for DCF *vs* 3.7 mo for PF, $P=0.0004$)^[58]. Overall survival was longer with DCF (risk reduction 23%, $P=0.02$). Another randomized phase II study conducted by the SAKK Co-operative Group, comparing DCF *versus* DC *versus* ECF concluded that DCF is the more promising schedule and should be chosen for a formal comparison with ECF in a phase III study^[59]. A recent three-arms randomized phase II study has compared LV 200 mg/m² (2-h infusion), followed by 5-FU 400 mg/m² bolus and 5-FU 600 mg/m² (22-h continuous infusion) on days 1 and 2, every 14 d (LV5FU2) *versus* LV5FU2 plus cisplatin 50 mg/m² or LV5FU2 plus CPT-11 180 mg/m², and showed that the overall response rates were 13%, 27% and 40%, respectively^[60]. The median progression-free survival (6.9 mo) and overall survival (11.3 mo) of the LV5FU2-irinotecan regimen were encouraging when compared with those of previous randomized studies. The accrual of a randomized phase III trial comparing irinotecan plus infusional 5-FU/LV *versus* cisplatin plus infusional 5-FU has been completed and the outcome is expected in the near future.

Finally, the ongoing REAL-2 study is testing the efficacy of oxaliplatin and capecitabine in a 2 \times 2 design. Six

Table 3 Meta-analysis of randomized trials of adjuvant cytotoxic chemotherapy in gastric cancer

| Author. ^{Year} | Number of patients/ number of trials included | Mortality risk ratio | 95% confidence interval | P value |
|----------------------------|---|-------------------------|----------------------------|---------|
| Hermans. ¹⁹⁹³ | 2 096/11 | 0.88 | 0.78-1.08 | NS |
| Earle. ¹⁹⁹⁹ | 1 190/13 | 0.80 | 0.66-0.97 | 0.024 |
| Mari. ²⁰⁰⁰ | 3 658/20 | 0.82 | 0.75-0.89 | <0.001 |
| Panzini. ²⁰⁰² | 2 913/17 | 0.72 | 0.62-0.84 | NA |
| Jannunger. ²⁰⁰² | 3 962/21 | 0.84 | 0.74-0.96 | NA |

hundred patients will be recruited and randomized to receive ECF *versus* EOF (epirubicin-oxaliplatin-5FU) *vs* ECX (epirubicin-cisplatin-capecitabine) *vs* EOX (epirubicin-oxaliplatin-capecitabine). An interim analysis presented at ASCO 2003 reported an interesting response rate of 52% for EOX compared with 31% for ECF, 33% for EOF and 35% for ECX^[61].

COMPLEMENTARY TREATMENT FOR LOCALIZED GASTRIC CANCER

Surgery remains the only potentially curative treatment, though it is associated with a high rate of locoregional failure. The surgical aim is the achievement of a curative resection (R0), which implies the removal of the primary tumour with clear resection margins and the removal of the regional lymph nodes^[62,63].

There is an ongoing debate whether more extensive lymph node dissection (D2) improves survival when compared to less extensive lymph node dissection. The D2 dissection was developed from surgical practice in Japan. Retrospective studies from Japan, involving more than 10 000 patients, suggested that D2 dissection prolonged survival compared to limited resection^[64-66]. However, two large phase III studies concluded that D2 dissection did not improve survival and was associated with higher surgical morbidity and mortality^[67,68]. The complication rate of D2 dissection was reported to be higher in the Dutch trial than in the Japanese experience. Further studies should be designed to evaluate the extended nodular dissection.

Nevertheless, the effectiveness of surgical resection is poor. R0 resection can be only achieved in 40% of the cases. Globally, almost 60% of patients who undergo a R0 resection will relapse and succumb to their disease; consequently the overall 5-year survival rate of patients with resectable gastric cancer ranges from 10% to 30%^[69,70]. The high risk of relapse after surgery has led to search strategies to prevent relapse and to improve survival for gastric cancer patients, as postoperative or adjuvant therapy strategies, or as preoperative or neoadjuvant approaches.

ADJUVANT CHEMOTHERAPY

The outcome of gastric cancer is related to a high

incidence of both local recurrence and distant metastases after curative surgery, and has led to major efforts to explore different adjuvant therapies, such as adjuvant systemic chemotherapy, adjuvant peritoneal chemotherapy or adjuvant immunochemotherapy.

Gastric cancer would seem to be an ideal setting to test adjuvant cytotoxic regimens, as there are multiple significantly active chemotherapy drugs and combinations that show antitumoral activity in the metastatic disease. However, over the last three decades multiple phase III studies, including an observational arm, have been reported and failed to demonstrate a clear improvement in survival, and therefore, this strategy is far to be the standard management following curative surgery.

Before 1993, there were no analyses supporting the regular use of adjuvant chemotherapy for gastric cancer^[71]. The minimal benefit of adjuvant cytotoxic chemotherapy alone has been published in several meta-analyses since 1993 (Table 3). The first meta-analysis published by Hermans *et al*^[72] in 1993 revealed no conclusive value for adjuvant cytotoxic chemotherapy. Later on, a meta-analysis published by Earle *et al*^[73] showed a marginal statistically significant, although clinically irrelevant, survival improvement for the adjuvant chemotherapy following surgical resection of gastric cancer. In 2000, Mari *et al*^[74] concluded that chemotherapy might offer a small survival advantage in patients with curatively resected gastric cancer, however, the same authors pointed that, in regard to the limitations of literature-based meta-analyses, adjuvant chemotherapy must be considered as an investigational approach. The meta-analysis reported by Panzini *et al*^[75] in 2002 showed that adjuvant chemotherapy resulted in a significant survival benefit in patients, though the authors suggested to confirm this conclusion in large prospective randomized trials. Finally, Jannunger *et al*^[76] in 2002 did not recommend adjuvant chemotherapy as routine therapy in this setting.

Overall, there is insufficient evidence nowadays to recommend postoperative chemotherapy as adjuvant treatment.

As systemic postoperative chemotherapy has not been accepted as standard treatment, other adjuvant strategies have been explored for patients with curatively resected gastric cancer, as adjuvant immunochemotherapy and adjuvant intraperitoneal therapy.

The adjuvant immunochemotherapy approach has been developed, fundamentally, in Korea and Japan. The benefit of a streptococcal preparation (OK-432) immunochemotherapy in patients with curatively resected gastric cancer was assessed by a meta-analysis involving 1 522 patients from six clinical trials^[77]. In these trials, chemotherapy was compared to the same chemotherapy plus immunotherapy. The 3-year survival rate was 67.5% in the immunochemotherapy group whereas 62.6% in the control group. The 3-year overall survival odds ratio was 0.81 (95% confidence interval: 0.65-0.99; $P=0.044$). The results of this meta-analysis suggested that immunochemotherapy with OK-432 after surgery may improve the survival of patients with curatively resected gastric cancer.

Intraperitoneal chemotherapy is a rational approach

Table 4 Phase II studies of preoperative chemotherapy in localized gastric cancer

| Author. ^{Year} | Number of patients | Neoadjuvant chemotherapy regimen | Adjuvant chemotherapy regimen | R0 rate | pCR | Median survival (mo) | 2-yr survival rate (%) |
|---------------------------|--------------------|----------------------------------|-------------------------------|---------|-----|----------------------|------------------------|
| Wilke. ¹⁹⁸⁹ | 34 | EAP×2 | EAP×2 | 29 | 5 | 18 | 26 |
| Leichman. ¹⁹⁹² | 38 | CF×2 | Intraperitoneal | 76 | 3 | 17 | NR |
| Ajani. ¹⁹⁹³ | 48 | EAP×3 | EAP×2 | 77 | 0 | 16 | 42 |
| Kang. ¹⁹⁹⁶ | 53 | EFP×2-3 | EFP×3-6 | 71 | 4 | 43 | NR |
| | 54 | Surgery | None | 61 | | 30 | NR |
| Crookes. ¹⁹⁹⁷ | 59 | CF×2 | Intraperitoneal | 71 | 5 | 48 | 64 |
| Songun. ¹⁹⁹⁹ | 27 | FAMtx×4 | None | 56 | 0 | 36 | 62 |
| | 29 | Surgery | None | 62 | | 13.1 | NR |

EAP: etoposide, doxorubicin and cisplatin; CF: cisplatin and 5-fluoruracil; EFP: etoposide, 5-fluorouracil and cisplatin; FAMtx: 5-fluorouracil, doxorubicin and methotrexate; pCR: pathological complete remission; NR: no reported

since the peritoneal cavity is the site of recurrence in more than half of the patients, and commonly the only one location of progression disease. In other tumours, such as ovarian cancer, in which peritoneal failure is common, clinical trials have demonstrated a small but significant advantage to intraperitoneal therapy^[78,79]. Nevertheless, the trials reported in gastric cancer have been hampered by small size and limited statistical power and have not established a clear survival benefit from this strategy^[80,82].

ADJUVANT CHEMORADIOTHERAPY

Taking into account that surgical resection of gastric cancer is curative in less than 40% of cases, and the fact that both locoregional and distant relapses are common, adjuvant chemoradiotherapy is a rational approach for these patients. Several preliminary studies have shown promising results^[83,84]. The most important trial in this setting is the US Intergroup 0116 phase III study. Five-hundred and fifty six patients with adenocarcinoma of the stomach and esophagogastric junction were randomized to surgery alone or surgery plus postoperative chemoradiation (1 cycle of 5-FU modulated by leucovorin, followed by concomitant 5-FU/LV and radiation therapy (45 Gy), and one month after the completion of radiotherapy, two 5-d cycles of 5-FU/LV)^[85]. Eighty-five percent of patients in both arms had node-positive carcinoma (stage III or IV). The toxicity in the study arm was: 41% of patients experienced grade III and 32% grade IV toxicity, with a 1% of toxic deaths. Median overall survival was significantly improved in the experimental arm compared to the surgery alone group (36 mo *vs* 27 mo; $P=0.005$). The 3-year overall survival (50% *vs* 41%) and 3-year relapse-free (48% *vs* 31%) survival were significantly better in the study group. The survival improvement was mostly related to the decrease in locoregional rather than distant failure. Based on the results of US Intergroup 0116 study, postoperative chemoradiation has been accepted as standard care in patients with resected gastric cancer in North America.

However, this study is limited for both the surgical procedures and the chemotherapy regimen employed. Fifty-four percent of patients underwent less than D1 dissection (only partial removal of the N1 nodes) and only

10% of patients underwent a D2 dissection. On the other hand, 5-FU/LV is not a chemotherapeutic option in the management of patients with advanced gastric cancer. The benefit of the Intergroup 0116 may have primarily derived from the use of radiation therapy, since local control was improved without any effect in distant control. These data support a potential benefit of this chemoradiation schedule after inadequate surgical resection. Further studies employing D1 or D2 dissection alone *versus* the same surgery plus adjuvant cisplatin/5-FU-based combinations plus radiation therapy are needed.

NEOADJUVANT CHEMOTHERAPY

Neoadjuvant treatment, chemotherapy or chemoradiotherapy, has been tested in small studies. Preoperative chemotherapy may allow to improve in the R0 rate due to tumour down-staging, and also may contribute to eradicate micrometastasis. At present, neoadjuvant treatment is being tested in locally advanced non-resectable tumours and in those resectable tumours with a high risk of recurrence.

However, there are possible disadvantages of the neoadjuvant treatment. Patients with early gastric carcinoma (stages 0 and I) could be over-treated, and among the stages II-IV non-metastatic gastric cancers, the response to the preoperative therapy could be unsatisfactory; therefore, some patients could be exposed to an unnecessary morbidity, and furthermore, the success of surgical resection could be hampered. In this context, efforts to identify prognostic factors and more active and less toxic preoperative regimens are being searched.

Several small phase II trials with different cisplatin-based neoadjuvant chemotherapy regimens have been reported in the nineties, with the largest including only 59 patients (Table 4). The perioperative radiotherapy was not included in any trials, while postoperative systemic or intraperitoneal chemotherapy was administered in some of these trials. The median survival ranged from 16 to 48 mo, and was significantly better in those patients who underwent an R0 resection^[86-91]. Beyond these small trials, phase III prospective clinical trials are needed.

The large phase III UK Medical Research Council Adjuvant Gastric Infusional Chemotherapy (MAGIC)

trial was the first well-powered phase III neoadjuvant chemotherapy study to assess the efficacy of perioperative chemotherapy^[92].

Five hundred and three patients with potentially resectable gastric cancer were randomized to both preoperative and postoperative EFC therapy chemotherapy *versus* surgery alone. EFC regimen consisted of epirubicin (50 mg/m²) and cisplatin (60 mg/m²) administered on day 1, and protracted venous infusion of 5-FU (200 mg/m² per d) on days 1 to 21, administered every 3 wk for three cycles before and after surgery. The results of this trial demonstrated statistically significant improvement of the study arm in disease-free survival (HR 0.7%, 95% confidence interval 0.56-0.88%) and a strong trend towards better overall survival compared to surgery alone (HR 0.8, 95% confidence interval 0.63-1.01). There were other important results from the MAGIC study. Although in the study group, the number of patients who underwent surgery was slightly lower (85% *vs* 92%), the rate of pathological complete response was significant better in this group (79% *vs* 69%, *P*=0.018), and the surgical morbidity and mortality were not compromised. Furthermore, there was a significant tumour down-staging.

However, it remains unclear how the neoadjuvant therapy may be integrated into the multimodality management of localized gastric cancer. Consequently, ongoing randomized controlled trials are evaluating this issue.

Meanwhile some clinicians may elect to offer patients preoperative chemotherapy. Taking into account, both INT-0116 study and MAGIC study, combining neoadjuvant and postoperative chemoradiation might result in improving the outcome of patients with resectable gastric cancer.

NEOADJUVANT CHEMORADIOTHERAPY

There are few phase I-II studies exploring the role of neoadjuvant chemoradiotherapy in resectable gastric cancer. In 2003, Roth *et al*^[93] reported promising results of a phase I-II study with preoperative platinum and 5-FU plus radiotherapy in this setting. They found a 5% pathological complete responses, 80% resection rate without increasing the surgical morbidity, and 71% of 2-year survival^[93]. Similar results were reported in a phase II study of MD Anderson group with 5-FU and concomitant preoperative radiotherapy^[94].

Other approach is the three-step therapy, involving preoperative chemotherapy, followed by concomitant chemoradiation and finally the surgical procedure. The MD Anderson group reported two phase II studies. In the former study, the patients received a combination of cisplatin and 5-FU, and then radiotherapy concomitant with 5-FU, achieving a 29% rate of pathological complete response and 80% rate of resectability^[95]. In the latter study, a combination of paclitaxel, cisplatin and 5-FU was used as neoadjuvant chemotherapy, followed by concomitant radiotherapy plus 5-FU. The resectability rate reported was 100%, with 26% of resections showing no viable tumour in the pathologic examination^[96]. Finally, some other trials are exploring other chemotherapeutic agents, such as docetaxel or irinotecan.

CONCLUSIONS OF COMPLEMENTARY TREATMENT FOR LOCALIZED GASTRIC CANCER

Gastric cancer is a disease in which locoregional control is hardly achieved. The roles of adjuvant and neoadjuvant therapy have also been debated for a long time; there are some approaches that seem to be effective for the management of localized gastric cancer.

Currently, it remains unclear to agree the standard care, and even it differs in each country. In EEUU, adjuvant chemoradiation is considered the standard care and stands for the control arm for further trials. However, in Korea, the standard care is adjuvant immunochemotherapy. In Japan, a D2 lymph node dissection is the standard surgical procedure and the role of any adjuvant chemotherapy is controversial.

In Europe, there are the most different opinions. Some authors argue that following an optimal surgery (at least D1), none of the adjuvant strategies seem to be superior to surgery alone, but other authors do agree to the INT-0116 results.

On the other hand, the MAGIC trial added important information on adjuvant strategy, though it is required to wait for definitive survival results of the MAGIC and other phase III trials, to accept neoadjuvant chemotherapy as the new standard care. In addition, neoadjuvant chemoradiotherapy trials offer promising results and these schemes may be explored in large randomized phase III trials. Newer approaches to cancer management, including molecular-targeted therapies may be explored.

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BASIC RESEARCH

1,2,3,4,6-penta-O-galloyl- β -D-glucose up-regulates heme oxygenase-1 expression by stimulating Nrf2 nuclear translocation in an extracellular signal-regulated kinase-dependent manner in HepG2 cells

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stimulating Nrf2 nuclear translocation in an ERK-dependent manner, and HO-1 expression by PGG may serve as one of the important mechanisms for its hepatoprotective effects.

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Abstract

AIM: To examine the potency of 1,2,3,4,6-penta-O-galloyl- β -D-glucose (PGG) as a hepatic heme oxygenase-1 (HO-1) inducer and its regulation in HepG2 cells.

METHODS: Expression of HO-1 and NF-E2-related factor 2 (Nrf2) and activation of mitogen-activated protein (MAP) kinases were analyzed by Western blot, immunofluorescence assay, and flow cytometry. Transfections of HO-1 gene, small interfering RNAs for HO-1 and Nrf2, and dominant-negative gene for MAP/extracellular signal-regulated kinase (ERK) were carried out to dissect the signaling pathways leading to HO-1 expression in HepG2 cells.

RESULTS: PGG up-regulated HO-1 expression and this expression conferred cytoprotection against oxidative injury induced by t-butyl hydroperoxide. Moreover, PGG induced Nrf2 nuclear translocation, which was found to be an upstream step of PGG-induced HO-1 expression, and ERK activation, of which pathway was involved in PGG-induced Nrf2 nuclear translocation, HO-1 expression and cytoprotection.

CONCLUSION: PGG up-regulates HO-1 expression by

INTRODUCTION

Oxidative stress is the inappropriate exposure to reactive oxygen species (ROS), such as superoxide anion radicals and hydroxyl radicals. High levels of ROS cause damage to cells and are involved in several human pathologies, including liver cirrhosis and fibrosis^[1,2]. Therefore, the use of compounds with antioxidant properties may help prevent or alleviate many diseases associated with ROS.

Because ROS formation is a naturally occurring process, the mammalian cells have developed several protective mechanisms to prevent ROS formation or to detoxify the ROS. These mechanisms employ molecules called antioxidants as well as protective enzymes^[3]. Among the various cytoprotective enzymes, heme oxygenase-1 (HO-1) is recently highlighted by virtue of its hepatoprotective roles^[4-6].

The inducible HO-1 is a rate-limiting enzyme in heme catabolism, leading to the formation of carbon monoxide, iron, and biliverdin. Biliverdin is subsequently converted to bilirubin, a potent endogenous anti-oxidant^[7]. There is a large body of evidence suggesting that HO-1 plays a key role in maintaining anti-oxidant homeostasis during cellular stress^[7-9]. The induction of HO-1 gene is primarily

regulated at the transcriptional level, and its inducibility by various inducers is linked to the transcription factor NF-E2-related factor 2 (Nrf2)^[9].

Under normal conditions, Nrf2 is sequestered in the cytoplasm by binding to Keep1, an actin-binding protein^[10]. Several stimuli cause the disruption of this complex, freeing Nrf2 for translocation to the nucleus and dimerization with basic leucine zipper transcription factors^[11,12]. The mechanism by which Nrf2 is liberated from the Keep1-Nrf2 complex remains to be established. However, recent studies have suggested that the Nrf2 nuclear translocation requires the activation of the mitogen-activated protein kinases (MAPKs)^[13].

The three major MAPK pathways are represented by kinase cascades leading to activation of extracellular signal-regulated kinase (ERK), p38, and c-Jun N-terminal kinase (JNK)^[14]. All three pathways appear to be involved to some extent in HO-1 expression and Nrf2 nuclear translocation in response to diverse stimuli^[15-20].

In view of the growing evidence that HO-1 provides hepatoprotection^[4,6], HO-1 expression by pharmacological modulator may represent a novel target for therapeutic intervention. Particularly, the identification of a non-cytotoxic inducer of HO-1 may maximize the intrinsic antioxidant potential of cells. Several antioxidants from plant origins have been reported to induce HO-1 expression in a variety of cells, including liver cells, and hence to resist to the oxidative stress^[15-17, 21-24].

1,2,3,4,6-penta-O-galloyl- β -D-glucose (PGG), a bioactive tannin, is wide-spread and can be found in many medicinal plants^[25]. Previously, we have demonstrated that PGG is a promising inducer of HO-1^[26]. In this study, we aimed at examining the potency of PGG as a hepatic HO-1 inducer and its regulation in HepG2 cells. In addition, we examined the hepatoprotective significance of PGG-induced HO-1 against oxidant toxicity.

MATERIALS AND METHODS

Cell culture

HepG2 cells were obtained from ATCC (Manassas, VA) and maintained as monolayer cultures in F12 medium supplemented with 100 mL/L fetal bovine serum (FBS), 1.7 mg/mL sodium bicarbonate, 0.1 unit/mL insulin, 0.5 x minimal essential medium amino acid, 100 units/mL penicillin, and 100 μ g/mL streptomycin.

Antibody, DNA and RNA

Antibodies to phospho (p)-ERK-1/2, p-JNK-1/2, p-p38, ERK-1/2, JNK-1/2, p38, and Nrf2, and small interfering (si) RNAs for HO-1 and Nrf2 plus siRNA transfection kit were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). FITC-conjugated secondary antibody was purchased from BD PharMingen (San Diego, CA). Antibodies to HO-1, tubulin, and lamin B were obtained from Cell Signaling Technology (Beverly, MA). cDNAs encoding constitutively active (CA) and dominant negative (DN) mutants of MEK1 were kindly provided by Dr. K. Y. Choi (Yonsei University, Seoul, Korea). HO-1 cDNA was a kind gift from Dr. A. M. K. Choi (University of Pittsburgh, Pittsburgh, PA).

PGG, inhibitors and reagents

PGG was isolated from the root of *Paeonia lactiflora*, as previously described^[27]. U0126 and zinc protoporphyrin IX (ZnPP) were obtained from Promega (Madison, WI) and Porphyrin Products (Logan, UT), respectively. SP600125 was purchased from Calbiochem (San Diego, CA). SB203580 and other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated by using TRI ReagentTM (Sigma-Aldrich). First-strand cDNAs were synthesized from RNA using ImProm-II RT system (Promega). Then, PCR with Taq DNA polymerase (Promega) was performed for 27 cycles, and PCR products were analyzed by electrophoresis. The primers used for PCR amplification were 5'-ATGGATGATGATATCGCCGCG-3' and 5'-TCTCCATGTCGTCCCAGTTG-3' (β -actin, 248 bp), as well as 5'-AAGATTGCCAGAAAGCCCTGGAC-3' and 5'-AACTGTCGCCACCAGAAAGCTGAG-3' (HO-1, 399 bp).

Western blotting analysis and HO activity

Cells were solubilized in ice-cold 10 g/L Triton X-100 lysis buffer supplemented with protease and phosphatase inhibitors. After 30 min on ice, the lysates were clarified by centrifugation and the protein concentration was determined. Proteins were resolved with SDS-PAGE, transferred to nitrocellulose membranes, and probed with specific antibodies (diluted 1:1 000), followed by incubation with secondary antibody (diluted 1:5 000) for specific band detection. HO-1 activity was determined at the end of each treatment as described in our previous study^[26].

Immunofluorescence assay

Cells were grown on Lab-Tek II chamber slides. The cells were fixed using formalin and permeabilized with cold acetone. Immunofluorescence was then performed using Nrf2 or HO-1 antibody and FITC-labeled secondary antibody. The cells were stained with DAPI (Sigma-Aldrich) to visualize the nuclei. A Zeiss Axiovert 200 fluorescent microscope fitted with a HV-Deo camera and the appropriate filters were used to capture the fluorescent images.

Flow cytometry

Cells were washed three times with PBS, fixed with 40 g/L paraformaldehyde and permeabilized with 1 g/L saponin. HO-1 expression was determined by intracellular staining with HO-1 antibody labeling FITC-conjugated secondary antibody. The stained cells were analyzed using a FACSVantage flow cytometry (BD Biosciences, Franklin Lakes, NJ).

Cell viability

Cell toxicity was estimated using the tetrazolium salt reduction test (MTT) after 4-h exposure to t-butyl hydroperoxide (t-Bt-HP; Sigma-Aldrich). After changing culture medium, MTT (5 mg/mL in PBS) was added to a plate. Cells were allowed to incubate for 3 h at 37°C in a

atmosphere containing 50 mL/L CO₂. After solubilization of deposited MTT with dimethyl sulfoxide, absorbance was measured at 540 nm in a microplate reader.

Transient transfection

HepG2 cells were plated in 6-well plates at a density of 1.5×10^5 cells/well. After 24 h of plating, cells were transfected with expression plasmids using LipofectAMINE 2000 (Invitrogen), following the manufacturer's instructions. Briefly, cell culture medium was changed to a fresh medium before each transfection, 0.5 μ g of pRSV- β -galactosidase plasmid was co-transfected for transfection efficiency normalization in each transfection, and total amount of DNA transfected in each well was adjusted to 4 μ g by using the empty vector pCDNA3.1. Cells were incubated with transfection mixture for 5 h and then cultured in fresh medium for an additional 36 h. Transfection for each siRNA was carried out with siRNA transfection kit (Santa Cruz Biotechnology), following the manufacturer's instructions. The transfected cells were grown for 36 h.

Statistical analysis

Results were expressed as means \pm SE. Student's t-test was applied for comparison of the data. $P < 0.05$ was considered statistically significant.

RESULTS

HO-1 mRNA and protein expressions by PGG

To determine the effects of PGG on HO-1 gene transcription, we used human HepG2 cells, which have been widely used in drug metabolism, chemoprevention and hepatoprotective studies^[28]. HO-1 mRNA expression was analyzed by RT-PCR after treatment with PGG (1-20 μ mol/L) for 4 h. HO-1 mRNA expression was increased in a dose-dependent manner (Figure 1A). No significant toxicity was found to the cells at these concentrations of PGG (data not shown). To further determine whether PGG could induce HO-1 protein expression, cells were treated with different doses of PGG for 12 h. Treatment with PGG increased HO-1 protein expression in a dose-dependent manner (Figure 1B). In addition, HO activity was also increased in a dose-dependent manner (Figure 1C). In HepG2 cells treated with 20 μ mol/L of PGG, HO-1 expression was increased in a time-dependent manner, reaching a maximum at 12 h, followed by a decrease at 24 h (Figure 1D).

Cytoprotection by PGG-induced HO-1 against oxidative injury

To examine whether HO-1 expression by PGG could cause HepG2 cells to become more resistant to oxidative injury, the cells were pre-treated with medium or PGG (5-20 μ mol/L) for 12 h. After changing culture medium, cells were exposed for 4 h to t-Bt-HP, a compound commonly used to induce oxidative stress in biological system^[29]. Exposure to 100 μ mol/L of t-Bt-HP resulted in a marked reduction of cell viability. Pre-incubation with PGG for 12 h diminished t-Bt-HP toxicity in a dose-dependent manner and increased cell viability by 80% (Figure 2A).

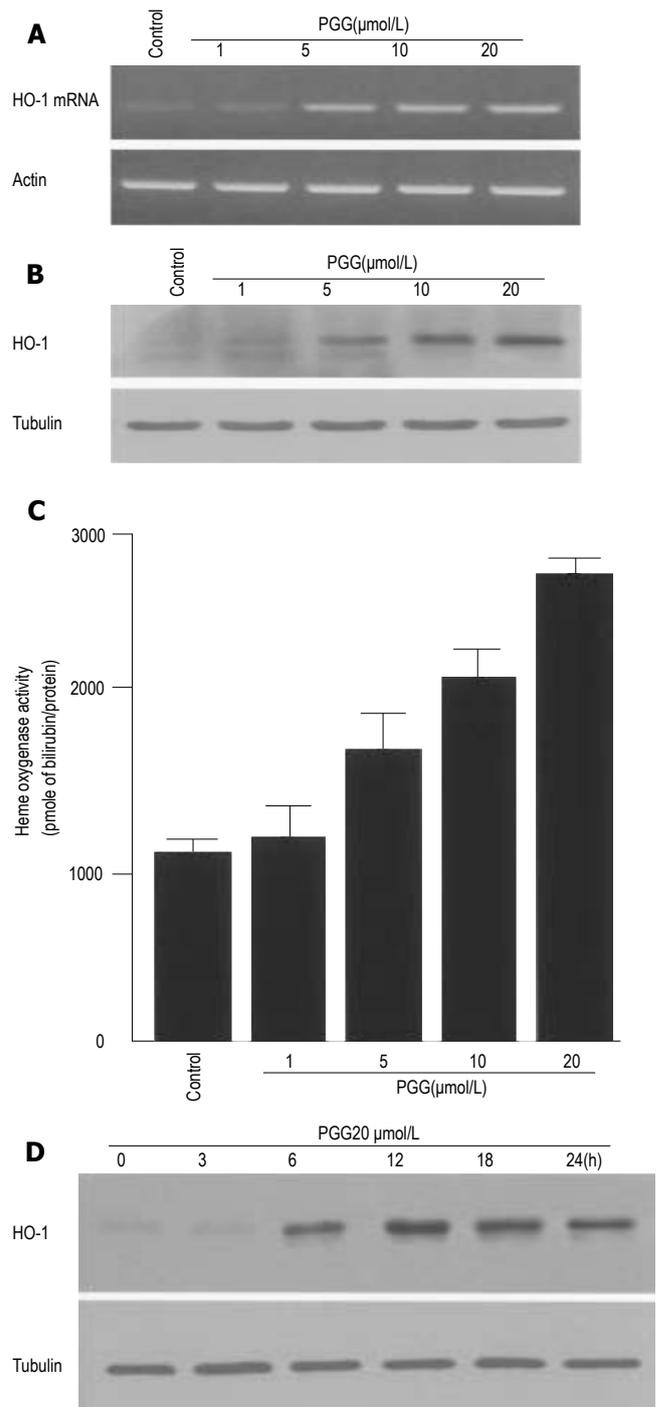


Figure 1 Effects of PGG on HO-1 mRNA and protein expression and HO activity in HepG2 cells. **A, B, C:** Cells were incubated with indicated concentrations of PGG for either 4 h (A) or 12 h (B and C); **D:** Cells were also incubated with 20 μ mol/L PGG for indicated times. Data are expressed as mean \pm SE of five independent experiments.

Cytoprotection by PGG was not detectable when the cells were transiently transfected with HO-1 siRNA (Figure 2A). Moreover, a similar cytoprotective effect was observed when the cells were transfected with HO-1 gene (Figure 2B). Blockage of the enzyme activity by ZnPP, a specific HO inhibitor, abolished HO-1-mediated cytoprotection (Figure 2B), suggesting that HO-1 metabolites could be of functional relevance of the observed protective action. Indeed, exogenously added bilirubin (0.1-10 μ mol/L),

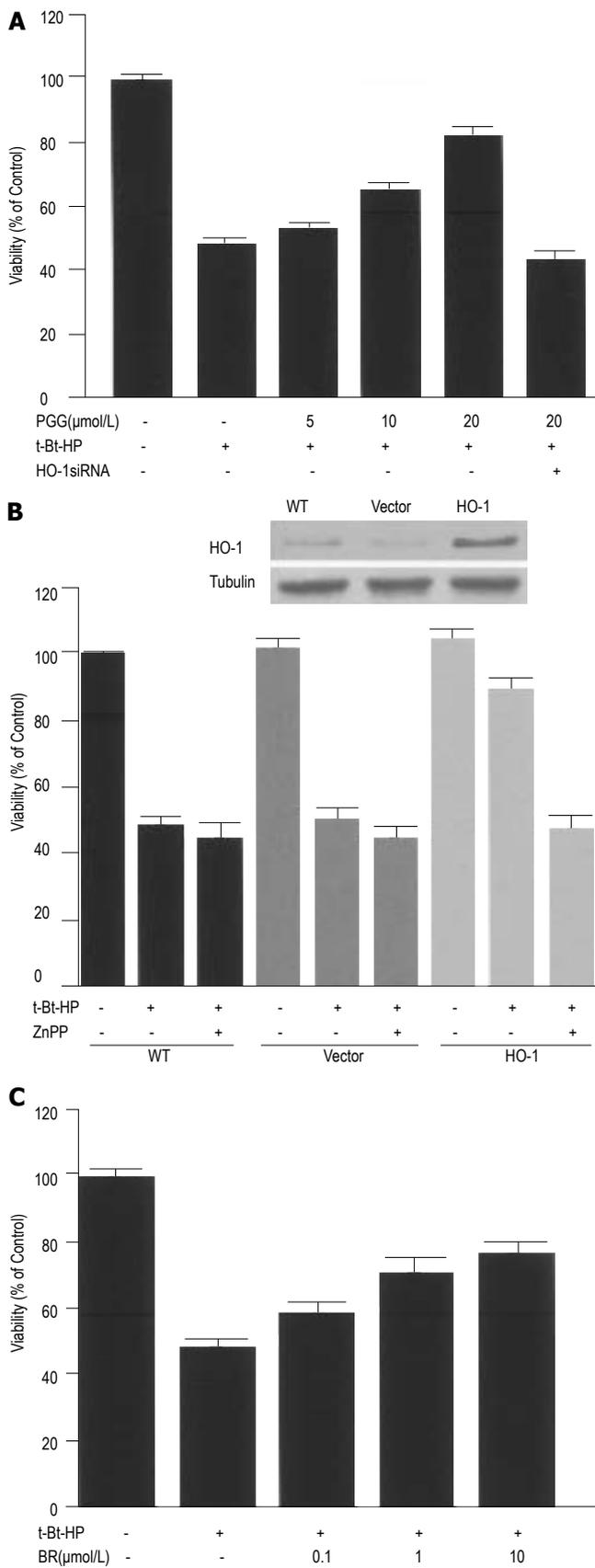


Figure 2 Effects of PGG-induced HO-1 on t-Bt-HP-induced toxicity in HepG2 cells. **A:** Cells transiently transfected with HO-1 siRNA were pre-incubated with indicated concentrations of PGG for 12 h, and exposed to 100 $\mu\text{mol/L}$ t-Bt-HP for 4 h; **B:** Cells (WT) were transiently transfected with control vector (Vector) or HO-1 gene (HO-1), and then exposed to 100 $\mu\text{mol/L}$ t-Bt-HP for 4 h in the absence or presence of 20 $\mu\text{mol/L}$ ZnPP; **C:** Cells were exposed to 100 $\mu\text{mol/L}$ of t-Bt-HP for 4 h in the absence or presence of indicated concentrations of bilirubin (BR). Data are expressed as mean \pm SE of five independent experiments.

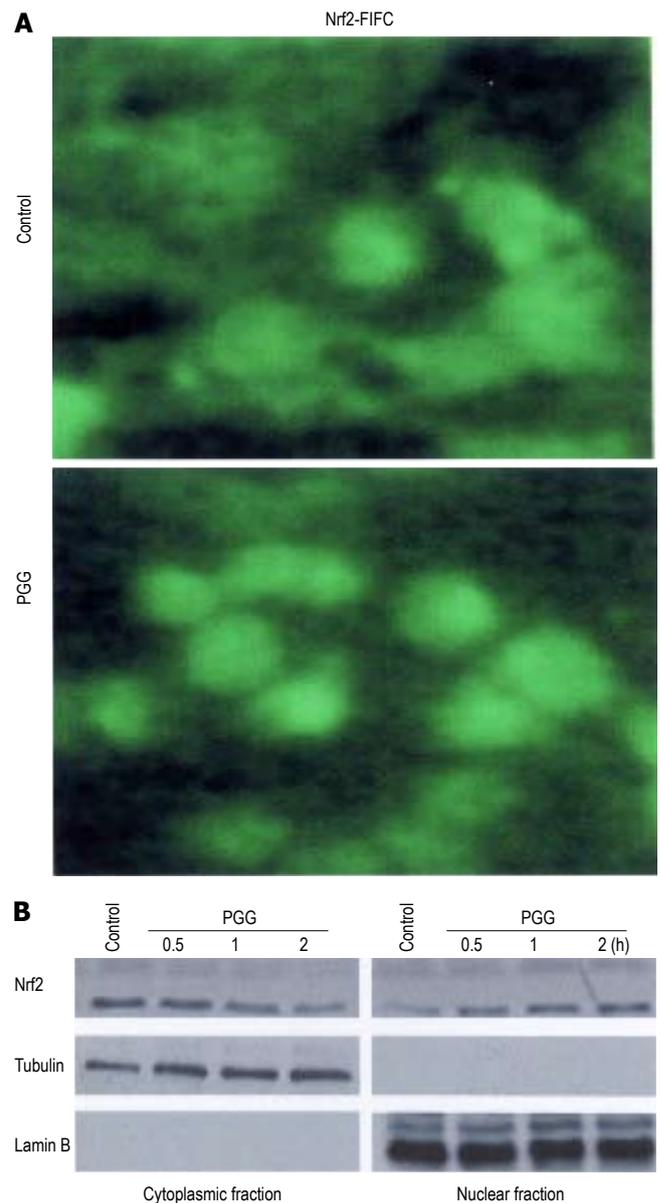


Figure 3 Effects of PGG on Nrf2 nuclear translocation in HepG2 cells. **A:** After 2-h treatment with 20 $\mu\text{mol/L}$ PGG; **B:** Cytoplasmic and nuclear extracts harvested from control cells and cells treated for indicated times with 20 $\mu\text{mol/L}$ PGG, and Western blotting performed with antibodies for Nrf2, tubulin and lamin B.

one of three HO-1 metabolites, produced a substantial reduction of t-Bt-HP toxicity (Figure 2C). HO-1 siRNA and ZnPP alone had no significant effect on cell viability under these conditions (data not shown).

Nrf2 nuclear translocation by PGG

In the mechanism of HO-1 expression, nuclear translocation of activated Nrf2 is an important upstream step^[9,30]. To investigate whether PGG could induce Nrf2 translocation in HepG2 cells, an immunofluorescence assay was used to detect the distribution of Nrf2 in the cells treated with medium or PGG (Figure 3A). Approximately 20 $\mu\text{mol/L}$ PGG was chosen to further determine the effects of PGG, because PGG at this concentration showed no significant toxicity (data not shown), caused a maximal increase in HO-1 expression (Figure 1) and also conferred a maximal cytoprotection (Figure 2). In untreated HepG2

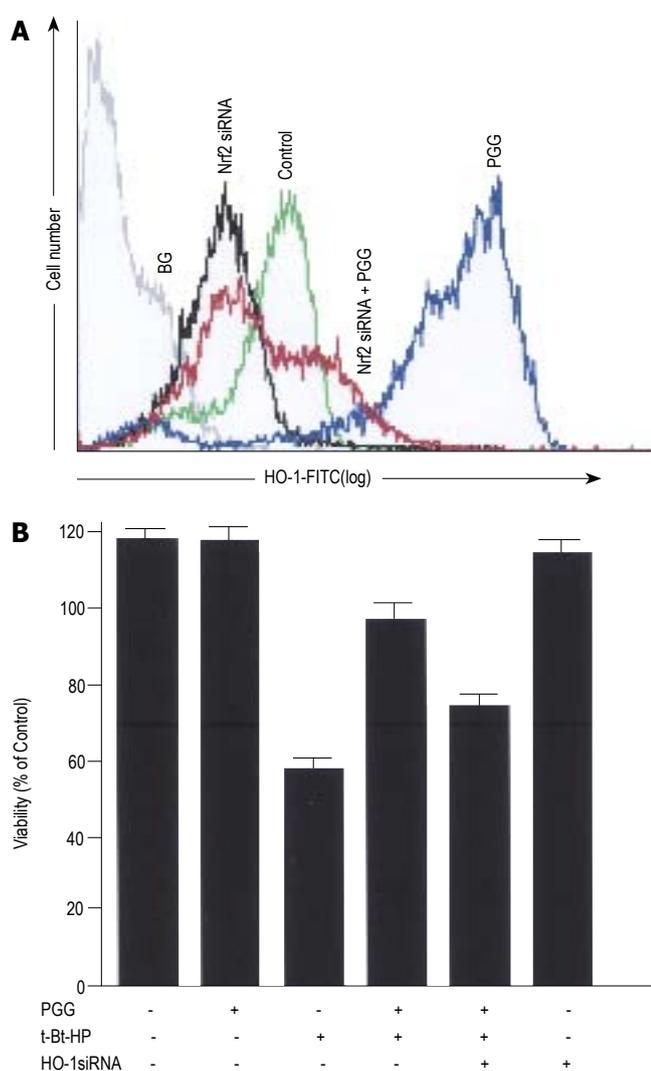


Figure 4 Effects of PGG-induced Nrf2 activation on HO-1 expression and t-Bt-HP-induced toxicity in HepG2 cells. **A:** Normal cells and the cells transiently transfected with Nrf2 siRNA were pre-incubated with 20 $\mu\text{mol/L}$ PGG for 12 h. HO-1 expression was determined by a flow cytometry. BG indicates background auto-fluorescence; **B:** Cells and transfected cells pre-incubated for 12 h with 20 $\mu\text{mol/L}$ PGG were exposed to 100 $\mu\text{mol/L}$ t-Bt-HP for 4 h. Data are expressed as mean \pm SE of five independent experiments.

cells, Nrf2 fluorescence was found to be distributed throughout the cells including cytoplasm and nucleus^[15]. After treatment with PGG for 2 h, Nrf2 fluorescence was primarily concentrated in the nuclei (Figure 3A). To further confirm the Nrf2 nuclear translocation by PGG, cells were incubated with PGG for about 2 h. Using Western blot, Nrf2 proteins in the cytoplasm and nuclear compartments of the cells were analyzed. After treatment with PGG, Nrf2 protein in the cytoplasm was decreased, and Nrf2 protein in the nucleus was markedly increased (Figure 3B). In addition, PGG-induced HO-1 expression was significantly reduced by transient transfection with Nrf2 siRNA (Figure 4A), as quantified by a flow cytometry, and cytoprotection afforded by PGG was effectively abolished by Nrf2 siRNA (Figure 4B).

Involvement of ERK MAPK pathway in HO-1 expression by PGG

MAPKs have been reported to participate in the regulation

of HO-1 expression^[15-20]. We examined the effects of PGG on MAPK activities in HepG2 cells. The cells were treated with medium or 20 $\mu\text{mol/L}$ PGG for about 2 h, and cell extracts were analyzed for phosphorylated and total MAPKs by Western blot. While the phosphorylated forms of all three MAPKs could be detectable even in the untreated cells, only the phosphorylated form of ERK was significantly increased by PGG (Figure 5A). Increased phosphorylation of ERK was detected within 30 min after treatment with PGG, and the amount of this kinase remained above the basal level for about 2 h. To address the role of individual MAPK pathway in HO-1 expression by PGG, we examined the effects of U0126, SB203580 and SP600125, specific inhibitors for ERK, p38 and JNK pathways, respectively, on HO-1 expression. Whereas inhibitor of either p38 or JNK had no effect on PGG-induced HO-1 expression, inhibitor for ERK MAPK pathway significantly reduced HO-1 expression (Figure 5B). In addition, we examined the effects of three MAPK inhibitors on PGG-induced cytoprotection. As expected, inhibitor for ERK MAPK pathway abolished PGG-induced cytoprotection, but inhibitors for p38 or JNK MAPK pathways did not (Figure 5C). The inhibitor for ERK alone had no significant toxicity under our experimental conditions (data not shown).

Involvement of ERK MAPK activation in Nrf2 nuclear translocation by PGG

It has been reported that MAPK-directed phosphorylation is a requirement for Nrf2 nuclear translocation in HepG2 cells^[31-33]. Thus, we examined whether MAPK pathway could be involved in the process by which PGG caused Nrf2 nuclear translocation. As shown in Figure 6A, inhibitor for ERK MAPK pathway, but not for p38 or JNK MAPK pathways, blocked PGG-induced Nrf2 nuclear accumulation. This was similar to the role of ERK inhibitor in PGG-induced HO-1 expression (Figure 5B) and cytoprotection (Figure 5C). To further evaluate the role of the ERK activation in PGG-induced Nrf2 nuclear translocation and HO-1 expression, we transfected DN-MEK1 and CA-MEK1 genes into HepG2 cells to selectively inhibit or activate the ERK pathway. In HepG2 cells, CA-MEK1 expression itself activated Nrf2 nuclear translocation and also HO-1 expression (Figure 6B). Moreover, PGG-induced cytoprotection (Figure 6C) and HO-1 expression (Figure 6D) were not observed when the cells were transfected with DN-MEK1 gene.

DISCUSSION

We previously reported that PGG, one of bioactive tannins, induced HO-1 expression^[26]. In this study, we aimed to examine the potency of PGG as a hepatic HO-1 inducer and its regulation in HepG2 cells. Our results showed that PGG induced HO-1 expression via stimulating Nrf2 nuclear translocation in an ERK-dependent manner, thereby exerting hepatoprotective effects.

The cytoprotective properties of antioxidants have been partially ascribed to their ability to induce cytoprotective enzymes. Among the various cytoprotective enzymes,

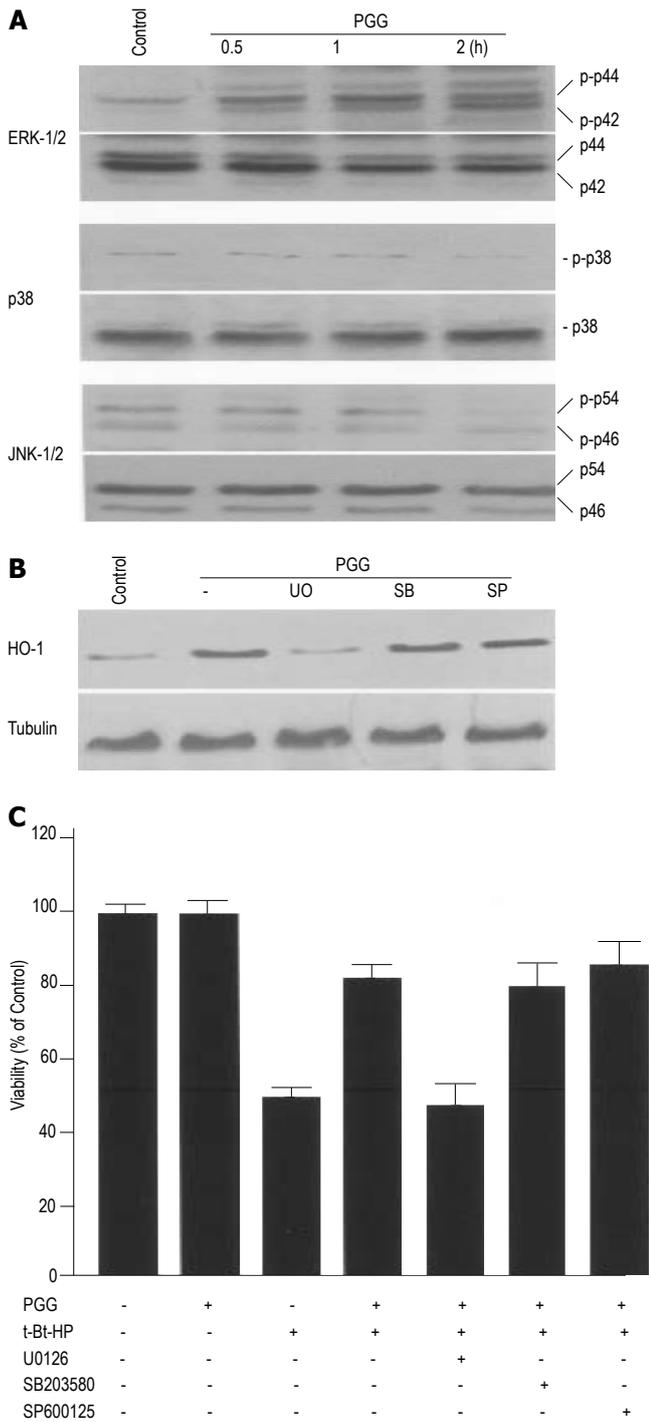


Figure 5 Effects of PGG-induced ERK activation on HO-1 expression and t-Bt-HP-induced toxicity in HepG2 cells. **A:** Cells were incubated with 20 $\mu\text{mol/L}$ PGG for indicated times, and Western blotting was performed with specific antibodies; **B:** Cells were incubated with 20 $\mu\text{mol/L}$ PGG for 12 h in the presence or absence of 10 $\mu\text{mol/L}$ U0123 (UO), 20 $\mu\text{mol/L}$ SB203580 (SB), or 20 $\mu\text{mol/L}$ SP600125 (SP), and Western blotting was performed with HO-1 antibody; **C:** Cells untreated or treated with PGG in the presence or absence of each specific inhibitor for 12 h were exposed to 100 $\mu\text{mol/L}$ t-Bt-HP for 4 h. Data are expressed as mean \pm SE of five independent experiments.

HO-1 expression has been considered to be an adaptive and beneficial response to oxidative stress in a wide variety of cells^[4-9]. Here, we demonstrated that PGG at non-cytotoxic concentrations increased HO-1 mRNA and protein expressions as well as HO activity in HepG2 cells (Figure 1). The increase of HO-1 expression by

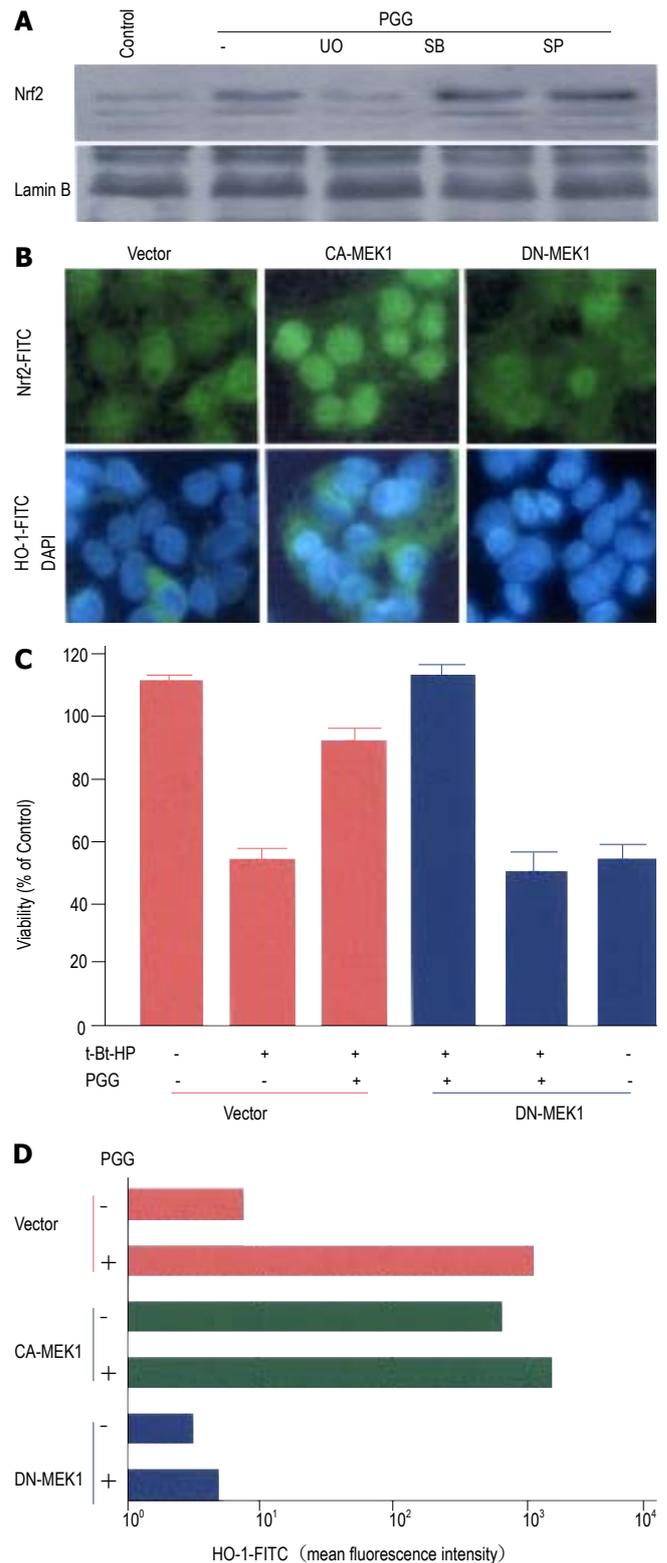


Figure 6 Roles of PGG-induced ERK activation in Nrf2 nuclear translocation and t-Bt-HP-induced toxicity in HepG2 cells. **A:** Cells were incubated with 20 $\mu\text{mol/L}$ PGG for 2 h in the presence or absence of 10 $\mu\text{mol/L}$ U0123 (UO), 20 $\mu\text{mol/L}$ SB203580 (SB), or 20 $\mu\text{mol/L}$ SP600125 (SP), and nuclear extracts were harvested, following Western blotting analysis with Nrf2 antibody; **B:** Cells were transiently transfected with control vector (Vector), CA-MEK1 gene, or DN-MEK1 gene, stained with Nrf2 antibody (upper) or HO-1 antibody and DAPI (lower), and visualized using a fluorescent microscope; **C:** Cells transfected with control vector or DN-MEK1 gene were pre-incubated with 20 $\mu\text{mol/L}$ PGG for 12 h, and exposed to 100 $\mu\text{mol/L}$ t-Bt-HP for 4 h. Data are expressed as mean \pm SE of five independent experiments; **D:** Cells transfected with vector or each gene were incubated for 12 h in the absence or presence of 20 $\mu\text{mol/L}$ PGG. HO-1 expression was determined by a flow cytometry.

PGG conferred cytoprotection against t-Bt-HP-induced oxidative stress (Figure 2A), which was clearly confirmed by transfection of either HO-1 siRNA (Figure 2A) or HO-1 gene (Figure 2B). We also demonstrated that cytoprotection afforded by HO-1 was highly dependent on its enzyme activity (Figure 2B) and bilirubin, one of its enzymatic products (Figure 2C). Our results suggested that PGG-induced HO-1 expression might serve as one of the important mechanisms for its hepatoprotective effects.

While the induction of HO-1 has been extensively reported and is known to be regulated primarily at the level of gene transcription, the molecular mechanism(s) underlying this response is poorly understood. However, recent evidences have implicated Nrf2 in inducer-dependent activation of the HO-1 gene^[9,30]. In our study, we found that PGG could activate Nrf2 in HepG2 cells. PGG increased not only Nrf2 nuclear translocation (Figure 3A), but also Nrf2 nuclear accumulation (Figure 3B). Using Nrf2 siRNA transfection method, we could show that the full expression of HO-1 by PGG required Nrf2 nuclear accumulation (Figure 4A). Along with this result, cytoprotection afforded by PGG was reduced by transient transfection with Nrf2 siRNA (Figure 4B). These results suggested that Nrf2 nuclear translocation might play a key role in PGG-induced HO-1 expression and cytoprotection.

MAPK pathways have been reported to be involved in HO-1 expression^[15,20] and also in Nrf2-dependent transcription^[31-33]. Our experiments designed to determine a possible role of MAPK pathways in PGG-induced HO-1 expression showed that PGG activated ERK pathway (Figure 5A). Additionally, the use of specific inhibitors for MAPK pathways confirmed the involvement of ERK, but not of p38 or JNK pathways, in PGG-induced HO-1 expression (Figure 5B) and cytoprotection (Figure 5C). These results suggested that ERK MAPK pathway might be important for PGG-induced HO-1 expression.

In light of our finding that ERK inhibitor blocked PGG-induced Nrf2 nuclear accumulation (Figure 6A), it is interesting to speculate that ERK pathway may play a role in regulating PGG-induced Nrf2 nuclear translocation. Our hypothesis is that addition of PGG may increase ERK activation in HepG2 cells, somehow to subsequently stimulate Nrf2 nuclear translocation. Increased Nrf2 in the nucleus may increase its DNA-binding activity, followed by HO-1 gene transcription eventually leading to HO-1 expression. This was confirmed by the transfection of HepG2 cells with CA-MEK1 or DN-MEK1 genes to selectively activate or inhibit the ERK pathway. Activation of the ERK pathway by CA-MEK1, even without further stimulation, was able to induce Nrf2-dependent HO-1 expression (Figure 6B). Blockage of the ERK pathway by DN-MEK1 abolished PGG-induced cytoprotection (Figure 6C) and HO-1 expression (Figure 6D). It is interesting that ERK activation is required for Nrf2 nuclear translocation during glutamate cysteine ligase modulatory gene expression by the antioxidant pyrrolidine dithiocarbamate in HepG2 cells^[32,33].

In summary, the antioxidant PGG can induce hepatic HO-1 expression in HepG2 cells, and this expression confers hepatoprotection against oxidative injury. PGG also induces Nrf2 nuclear translocation, which is an

upstream step of PGG-induced HO-1 expression, and activates ERK MAPK phosphorylation. ERK MAPK pathway is involved in PGG-induced Nrf2 nuclear translocation, HO-1 expression, and hepatoprotection. Thus, we speculate that PGG may be developed as a hepatic HO-1 inducer for therapeutic purposes. Despite the fact that up-regulation of HO-1 is sufficient to produce many beneficial outcomes in a variety of stressful condition^[4-9], we do not exclude the possibility that PGG will stimulate the expression of other defensive enzymes, probably depending on Nrf2 nuclear translocation, and that cellular and tissue protection will be achieved by virtue of the concerted action of the multiple pathways being activated.

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BASIC RESEARCH

Peanut consumption and reduced risk of colorectal cancer in women: A prospective study in Taiwan

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CONCLUSION: This study suggests that frequent intake of peanut and its products may reduce colorectal cancer risk in women, demonstrating the anti-proliferating effect of peanut intake.

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Key words: Peanuts; Colorectal cancer; Diet; Gender difference; Prospective study; Taiwan

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Abstract

AIM: To examine whether peanut consumption is associated with a reduced risk of colorectal cancer in a prospective cohort with a 10-year follow-up.

METHODS: In 1990-1992, residents (12 026 men and 11 917 women aged 30 to 65 years) in 7 townships, Taiwan, were interviewed and recruited into a cancer-screening cohort and annually followed up. Colorectal cancer cases in this cohort were identified from cancer registry and death certificates. Incidence rates of this disease by the end of 2001 were calculated by gender for the primary study variable and covariates. The dietary intake was assessed by means of weekly food frequency measures, including frequently consumed food groups and folk dishes including sweet potato, bean products, peanut products, pickled foodstuffs, nitrated or smoked foodstuffs.

RESULTS: During the study period, 107 new colorectal cancer cases (68 men and 39 women) were confirmed. The multivariate Cox's proportional hazard model showed that the relative risk (RR) of peanut consumption was 0.73 [95% confidence interval (CI) = 0.44-1.21] for men and 0.42 (95% CI = 0.21-0.84) for women. However, frequent intake of pickled foodstuffs was harmful for women (RR = 2.15, 95% CI = 0.99-4.65). The risk of colorectal cancer was also elevated among cigarette smokers but not significant ($P < 0.05$).

INTRODUCTION

Etiological studies showed that more than 85% colorectal cancer cases are associated with environmental factors^[1,2], particularly the dietary factor^[3]. Among dietary constituents implicating in colorectal carcinogenesis, consumption of vegetables and fruits has a beneficial effect^[4]. But the effect of pulse (legume) consumption is conflicting^[5]. An ecological study reported consumption of pulses is inversely related with colon cancer mortality of males in 38 countries (correlation coefficient = -0.7)^[6]. Previous studies showed that components, such as folic acid, protease inhibitors, phytosterols (PS), saponins, inositol hexaphosphate (phytic acid, PA), isoflavones and resveratrol in grains, nuts and seeds may have anti-carcinogenic effects^[7,8]. Several studies reported consumption of pulses is inversely related with colorectal cancer^[9-12], but other studies have failed to show this protective effect^[13-16]. As a good source of PS, PA and resveratrol, peanut has been thought to have an effect against carcinogenesis^[17]. The suppression effect of PS, PA and resveratrol on colon cancer has been supported *in vitro* and *in vivo* as well^[18-20]. Whether the laboratory findings are relevant to the hypothetically protective effect of peanut on cancers remains unexplored.

Colorectal cancer has become the second most fatal malignancy in developed countries and the third most frequent cancer worldwide^[21,22]. While the incidence rate of colorectal cancer also increases in Taiwan, the mortality rate of the disease has a 74% rise from 1993 to 2002.

Table 1 Incidence and relative risk of colorectal cancer by social demographic characteristics in a Taiwan community-based cancer screening cohort (1991-2001)

| Characteristic | Subject number | Person-yr | Case number | Colorectal cancer | | |
|---------------------|----------------|-----------|-------------|-------------------|--|-------|
| | | | | Rate ¹ | RR ² (95% CI ³) | P |
| Age (yr) | | | | | | |
| <50 | 12902 | 129055 | 18 | 13.9 | 1.00 | |
| 50-59 | 6679 | 66812 | 51 | 76.3 | 5.47 (3.20-9.36) | <0.01 |
| +60 | 2534 | 24944 | 38 | 152.0 | 11.0 (6.30-19.4) | <0.01 |
| Gender | | | | | | |
| Women | 11192 | 112007 | 39 | 34.8 | 1.00 | |
| Men | 10923 | 108805 | 68 | 62.5 | 1.81 (1.22-2.68) | <0.01 |
| Education (yr) | | | | | | |
| None | 4576 | 45806 | 30 | 65.5 | 1.00 | |
| ≤6 | 9126 | 91009 | 56 | 61.5 | 0.94 (0.61-1.47) | 0.79 |
| >6 | 8383 | 83698 | 21 | 25.1 | 0.39 (0.22-0.67) | <0.01 |
| Unknown | 30 | 300 | 0 | — | — | |
| Ethnic group | | | | | | |
| Fukien | 14938 | 149709 | 73 | 48.8 | 1.01 (0.67-1.52) | 0.96 |
| Others ⁴ | 7128 | 70612 | 34 | 48.2 | 1.00 | |
| Unknown | 49 | 491 | 0 | — | — | |
| Residential area | | | | | | |
| Potzu | 3362 | 33680 | 23 | 68.3 | 1.51 (0.95-2.39) | 0.08 |
| Other ⁵ | 18753 | 187132 | 84 | 44.9 | 1.00 | |
| Married | | | | | | |
| Yes | 20354 | 203204 | 99 | 48.7 | 1.00 | |
| No | 1671 | 16699 | 8 | 47.9 | 0.98 (0.48-2.02) | 0.97 |
| Unknown | 90 | 909 | 0 | — | — | |
| All | 22115 | 220812 | 107 | 48.5 | | |

¹Rate in 10⁵ persons/year; ²Relative risk; ³95% Confidence interval; ⁴Including mainland Chinese, Hskka and aborigines; ⁵Including Sanchi, Chutung, Kaoshu, Makung, Hushi and Paihsa.

Peanut is a common food in the world and in Taiwan as well. The association between peanut consumption and colorectal cancer has not been well studied. The purpose of this study was to determine the association between peanut consumption and colorectal cancer risk in a community-based cancer-screening cohort with a 10-year follow-up.

MATERIALS AND METHODS

Cohort recruitment and inclusion criteria

The details of this prospective cohort study conducted in 7 townships (Sanchi, Chutung, Potzu, Kaoshu, Makung, Hushi and Paihsa) and their precincts, Taiwan, have been described elsewhere^[23]. A total of 23943 persons (12026 men and 11917 women) who were at least 30 years old and gave their consent to a baseline interview in person, physical examinations and annual follow-up activities, were recruited between July 1990 and June 1992 in this cohort study. At the same time, we conducted the laboratory tests of serum cholesterol and triglyceride for all participants.

Questionnaire interviews

Well-trained public health nurses employed at local health stations in the study townships assisted to conduct home visits to all participants. With consent, structured questionnaire interviews were conducted for information regarding socio-demographic characteristics (age, gender, education level, ethnicity group, residential area, and

marital status), anthropometric measures (height and weight), cigarette and alcohol use, dietary consumption, personal and family history of cancers. Dietary intake was assessed by means of weekly food frequency measures (0-1, 2-3, and >3 meals/week), including frequently consumed food groups and folk dishes including sweet potato, bean products, peanut products, pickled foodstuffs, nitrated or smoked foodstuffs.

Cigarette smoking and alcohol drinking were evaluated by their amount and duration. Total cigarette smoking was estimated in terms of packs/year (packs per day multiplying the years of cigarette smoking in the follow-up). Alcohol drinking in data analyses used a dichotomous classification: yes versus no. Height and weight were measured once at the baseline. This study also collected information on reproductive health, including the use of oral contraceptives or intrauterine devices (never or ever use) and menopausal status (yes or no) for females.

Follow-up and data linkage

All participants were annually followed up for their health status. In addition to the active follow-up by home visit or telephone interviews, data from the National Cancer Registry and death certificates were also used to identify and verify newly developed colorectal cancer and other types of cancer. Diagnoses of colorectal cancer were confirmed by reviewing the medical records and death certificates. Colon and rectal cancer cases were combined in this study because of the small sample size and potential for their mutual misclassification in some death certificates^[24].

Statistical analysis

After exclusion of the participants with cancer occurred prior to the recruitment ($n=1336$) or cancer cases ($n=492$) other than colorectal cancer, a cohort of 22115 (92%) persons remained for the present study analysis. In addition to calculation of the incidence of the disease, the Cox's proportional hazard model was used to estimate the relative risk (RR) and 95% confidence interval (CI) associated with socio-demographic factors, cigarette and alcohol use, body mass index (BMI), cholesterol, triglyceride and diet. The association with menopause was also considered for women. None of the female cases was a smoker or a alcohol user, the RR due to these two factors was calculated only for men. Because of the limited sample size, diet consumptions and other covariates were mostly categorized into two or three strata in the data analysis. BMI was categorized into four strata (<24.2, 24.2-26.4, 26.4-28.2, >28.2 kg/m²). For incidence and RR calculation, the number of persons/year under observation was computed for each stratum of categorized risk factors. The dose-response relationship across the level of life style variables was observed by treating each ordinal-score variable as a continuous variable in proportional hazard regression. A score selection procedure (the highest likelihood score statistic) was used to determine the covariates included in the final multivariate Cox's proportional hazard model beginning with all lifestyle and dietary variables. All analyses were performed using the SAS version 8.2 software (SAS Institute, Inc., Cary, North Carolina). All statistical tests were two-sided.

Table 2 Incidence of colorectal cancer and relative risk by lifestyle factor in a Taiwan community-based cancer screening cohort among men (1991-2001)

| Characteristic | Cases number | Rate ¹ | RR ² | 95% CI ³ | P |
|--------------------------------------|--------------|-------------------|-----------------|---------------------|-------|
| BMI (kg/m ²) | | | | | |
| <24.2 | 33 | 54.7 | 1.00 | | |
| 24.2-26.4 | 16 | 62.5 | 1.15 | 0.63-2.08 | 0.65 |
| 26.4-28.6 | 11 | 73.5 | 1.35 | 0.68-2.67 | 0.39 |
| 28.6+ | 8 | 101.4 | 1.87 | 0.86-4.04 | 0.11 |
| P for trend | | | 0.11 | | |
| Cholesterol (tertile ⁴) | | | | | |
| 1 | 15 | 41.5 | 1.00 | | |
| 2 | 18 | 49.5 | 1.19 | 0.60-2.36 | 0.62 |
| 3 | 35 | 96.5 | 2.32 | 1.27-4.25 | <0.01 |
| P for trend | | | 0.004 | | |
| Triglyceride (tertile ⁵) | | | | | |
| 1 | 20 | 54.2 | 1.00 | | |
| 2 | 21 | 58.4 | 1.07 | 0.58-1.98 | 0.83 |
| 3 | 27 | 75.1 | 1.38 | 0.78-2.46 | 0.27 |
| P for trend | | | 0.26 | | |
| Cigarette smoking (pack-yr) | | | | | |
| 0 | 22 | 45.2 | 1.00 | | |
| 1-25 | 21 | 56.7 | 1.26 | 0.69-2.28 | 0.46 |
| 26+ | 24 | 105.0 | 2.32 | 1.30-4.13 | <0.01 |
| Unknown | 1 | 896.0 | — | | |
| P for trend | | | 0.006 | | |
| Alcohol drinking | | | | | |
| No | 52 | 59.9 | 1.00 | | |
| Yes | 16 | 73.8 | 1.23 | 0.71-2.16 | 0.46 |

¹Incidence rate per 10⁵ persons/year; ²Relative risk; ³95% Confidence interval; ⁴The tertile of cholesterol level (mg/dL) was <163, 163-197, 197+; ⁵The tertile of triglyceride level (mg/dL) was <87, 87-145, 145+.

RESULTS

A total of 107 new cases of colorectal cancer (56 in the colon, 51 in the rectum) were found during the 10-year follow-up period until December 31, 2001. Table 1 shows the incidence rate of 34.8 per 10⁵ persons/year for women and 62.5 per 10⁵ persons/year for men. Most participants who received only elementary education or no school education had a greater incidence than persons who received 6 or more years of education. There were no significant relationships between colorectal cancer and ethnicity and marital status. Residents living in Potzu had a higher risk for the disease than those living in other areas but not significant ($P = 0.08$).

The incidence of the disease was positively associated with BMI, serum cholesterol and cigarette smoking in men ($P < 0.01$, Table 2). The incidence increased from 54.7 per 10⁵ persons/year in men with the BMI less than 24.2 kg/m² to 101.4 per 10⁵ persons/year for those with the BMI greater than 28.6 kg/m². In women, the incidence rate of colorectal cancer was related with their BMI, cholesterol and triglyceride level ($P < 0.05$, Table 3). The highest incidence rate in women was 70.2 per 10⁵ persons/year for those with their BMI being 26.4-28.6 kg/m². The results also showed that menopausal women had an increased risk for the disease with an incidence rate of 67.6 per 10⁵ persons/year.

The analysis of food intake showed that the incidence rate of colorectal cancer was significantly increased in men

Table 3 Incidence of colorectal cancer and relative risk by lifestyle factor in a Taiwan community-based cancer screening cohort among women (1991-2001)

| Characteristic | Cases number | Rate ¹ | RR ² | 95% CI ³ | P |
|--------------------------------------|--------------|-------------------|-----------------|---------------------|-------|
| BMI (kg/m ²) | | | | | |
| <24.2 | 12 | 19.3 | 1.00 | | |
| 24.2-26.4 | 12 | 50.3 | 2.61 | 1.17-5.80 | 0.02 |
| 26.4-28.6 | 10 | 70.2 | 3.64 | 1.57-8.43 | <0.01 |
| 28.6+ | 5 | 42.4 | 2.19 | 0.77-6.23 | 0.14 |
| P for trend | | | 0.012 | | |
| Cholesterol (tertile ⁴) | | | | | |
| 1 | 8 | 21.6 | 1.00 | | |
| 2 | 10 | 26.4 | 1.22 | 0.48-3.10 | 0.67 |
| 3 | 21 | 56.8 | 2.64 | 1.17-5.95 | 0.02 |
| P for trend | | | 0.012 | | |
| Triglyceride (tertile ⁵) | | | | | |
| 1 | 8 | 21.4 | 1.00 | | |
| 2 | 13 | 34.2 | 1.60 | 0.66-3.86 | 0.30 |
| 3 | 18 | 49.2 | 2.30 | 1.00-5.28 | 0.05 |
| P for trend | | | 0.046 | | |
| Oral contraceptives | | | | | |
| Never use | 27 | 38.2 | 1.00 | | |
| Ever use | 7 | 21.7 | 0.57 | 0.25-1.31 | 0.19 |
| Unknown | 5 | 54.9 | — | | |
| Intrauterine devices | | | | | |
| Never use | 15 | 35.8 | 1.00 | | |
| Ever use | 19 | 31.2 | 0.87 | 0.44-1.72 | 0.69 |
| Unknown | 5 | 54.5 | — | | |
| Menopause | | | | | |
| No | 10 | 14.9 | 1.00 | | |
| Yes | 24 | 67.6 | 4.53 | 2.17-9.47 | <0.01 |
| Unknown | 5 | 53.5 | — | | |

¹Incidence rate per 10⁵ persons/year; ²Relative risk; ³95% Confidence interval; ⁴The tertile of cholesterol level (mg/dL) was <165, 165-199, 199+; ⁵The tertile of triglyceride level (mg/dL) was <75, 75-126, 126+.

who had more sweet potatoes and in women who had more pickled foodstuffs (Table 4). Beneficial effects were of the consumption of peanut products observed in both sexes but not significant. There were no significant associations between the colorectal cancer risk and the consumption of bean products, nitrate and smoked foodstuffs in both sexes.

The score selection in the final multivariate Cox's proportional hazard analysis was completed with 5 final covariates including age, resident area, cigarette smoking, consumption of peanut products and BMI, which were potentially associated with the risk of the disease in men. For women, three potential covariates including age, consumption of peanut products and pickled foodstuffs were selected. Among these covariates, age was a significant factor for colorectal cancer in both sexes. Obese men living in Potzu had an increased risk for colorectal cancer. The RR was 1.98 (95% CI = 0.91-4.30) for the highest level of BMI (>28.6 kg/m² vs <24.2 kg/m²). Men who smoked cigarettes had an excess risk of 77% over non-smokers (RR = 1.77, 95% CI = 0.97-3.26). Women who had peanut products demonstrated a significant beneficial effect. The RR was 0.42 (95% CI = 0.21-0.84) for consuming 2+ meals/week compared with 0-1 meals/week. On the other hand, consumption of pickled foodstuffs also had a harmful effect on female colorectal cancer patients with a boarder line significance level (RR for +2 meals/week

Table 4 Incidence of colorectal cancer and relative risk associated with dietary factors in a Taiwan community-based cancer screening cohort (1991-2001)

| Food variable (meals/wk) | Men | | | Women | | |
|-----------------------------|-------------------------------|-------------------------------|------|-------------------------------|-------------------------------|------|
| | Cases (Rate ¹) | RR2 (95% CI ³) | P | Cases (Rate ¹) | RR2 (95% CI ³) | P |
| Folk Vegetarian | | | | | | |
| No | 68 (63.9) | 1.00 | | 37 (34.1) | 1.00 | |
| Yes | 0 | | | 2 (67.4) | 1.98 (0.48-8.22) | 0.35 |
| Unknown | 0 | — | | 0 | — | |
| Sweet potato | | | | | | |
| 0-1 | 8 (31.7) | 1.00 | | 8 (26.0) | 1.00 | |
| 2+ | 60 (72.3) | 2.26 (1.08-4.73) | 0.03 | 31 (38.4) | 1.48 (0.68-3.22) | 0.32 |
| Unknown | 0 | — | | 0 | — | |
| Bean products | | | | | | |
| 0-1 | 24 (60.0) | 1.00 | | 13 (27.1) | 1.00 | |
| 2+ | 43 (63.0) | 1.04 (0.63-1.71) | 0.88 | 26 (41.0) | 1.51 (0.78-2.94) | 0.23 |
| Unknown | 1 | — | | 0 | — | |
| Peanut products | | | | | | |
| 0-1 | 23 (70.8) | 1.00 | | 17 (40.4) | 1.00 | |
| 2+ | 44 (58.1) | 0.83 (0.50-1.37) | 0.45 | 21 (30.4) | 0.75 (0.40-1.43) | 0.38 |
| Unknown | 1 | — | | 1 | — | |
| Pickled foodstuffs | | | | | | |
| 0-1 | 26 (68.9) | 1.00 | | 10 (21.2) | 1.00 | |
| 2+ | 41 (58.2) | 0.84 (0.51-1.37) | 0.48 | 29 (45.2) | 2.13 (1.04-4.37) | 0.04 |
| Unknown | 1 | — | | 0 | — | |
| Nitrated foodstuffs | | | | | | |
| 0-1 | 59 (61.5) | 1.00 | | 34 (33.4) | 1.00 | |
| 2+ | 6 (53.5) | 0.87 (0.38-2.01) | 0.74 | 4 (44.1) | 1.32 (0.47-3.71) | 0.60 |
| Unknown | 3 | — | | 1 | — | |
| Smoked foodstuffs | | | | | | |
| 0-1 | 60 (60.5) | 1.00 | | 36 (34.1) | 1.00 | |
| 2+ | 5 (65.1) | 1.08 (0.43-2.69) | 0.87 | 2 (38.2) | 1.12 (0.27-4.64) | 0.88 |
| Unknown | 3 | — | | 1 | — | |

¹Incidence rate per 10⁵ persons/year; ²Relative risk; ³95% Confidence interval.

versus 0-1 meals/week was 2.15, 95% CI=0.99-4.65) (Table 5).

DISCUSSION

The present study had several weaknesses due the relatively short follow-up time (10 years), the lack of detailed information on meat, vegetables and fruits consumption, physical activity, family history of colorectal neoplasm, aspirin and other non-steroidal anti-inflammatory drug use, colon-cancer screening practices and the relatively small number of cases for a cohort study. However, this well-defined population-based prospective approach reduced the selection bias and strengthened the complete ascertainment of cancer cases in the study.

It was reported that colorectal cancer is related with the consumption of vegetables and fruits and that the intake of vegetables and fruits can moderately lower the risk for colorectal cancer^[5,25,26]. This 10-year follow-up study was not able to examine the effect of the consumption of vegetables and fruits. However, our results support the hypothesis that the consumption of peanut protects those who consume peanut products against colorectal cancer. This finding indicates that some specific components in peanut inhibit the carcinogenic mechanisms of colorectal cancer. Peanut is a good source of phytosterols (PS)^[17], phytic acid (PA)^[17,19,27] and resveratrol^[28], which is con-

Table 5 Relative risk from multivariate analysis by Cox's proportional hazard model of the community-based cancer screening cohort (1991-2001)

| Variables | Men | | | Women | | |
|-----------------------------|-----------------|---------------------|-------|------------------|---------------------|-------|
| | RR ¹ | 95% CI ² | P | RR ¹ | 95% CI ² | P |
| Age (yr) | 1.13 | 1.09-1.17 | <0.01 | 1.1 ² | 1.08-1.17 | <0.01 |
| Residential area | | | | | | |
| Potzu | 2.13 | 1.24-3.68 | <0.01 | NI ⁴ | | |
| Other ³ | 1.00 | | | | | |
| Cigarette smoking (pack-yr) | | | | | | |
| 0 | 1.00 | | | NI ⁴ | | |
| 1-25 | 1.77 | 0.97-3.26 | 0.06 | | | |
| 26+ | 1.51 | 0.83-2.74 | 0.18 | | | |
| Peanut products | | | | | | |
| 0-1 | 1.00 | | | 1.00 | | |
| 2+ | 0.73 | 0.44-1.21 | 0.22 | 0.42 | 0.21-0.84 | 0.01 |
| Pickled foodstuffs | | | | | | |
| 0-1 | NI ⁴ | | | 1.00 | | |
| 2+ | | | | 2.15 | 0.99-4.65 | 0.05 |
| BMI (kg/m ²) | | | | | | |
| <24.2 | 1.00 | | | NI ⁴ | | |
| 24.2-26.4 | 1.19 | 0.65-2.17 | 0.58 | | | |
| 26.4-28.6 | 1.26 | 0.62-2.57 | 0.53 | | | |
| 28.6+ | 1.98 | 0.91-4.30 | 0.09 | | | |

¹Relative risk; ²95% Confidence interval; ³Including Sanchi, Chutung, Kaoshu, Makung, Hushi and Paihsa; ⁴Not included in the model.

sumed more in Asians (particularly in vegetarians) than in Westerns^[7]. PS consists of approximately 80% β -sitosterol and 20% campesterol and stigmasterol^[17]. β -sitosterol is the main component offering the protective effect against colon cancer by inhibiting HT-29 colon cancer cells^[29]. The consumption of PS inhibits the development of chemically-induced colon tumors in rats^[18]. In addition, PS consumption normalizes the hyper-proliferating state in rat and mouse colon cells^[30].

PA consisting of a myo-inositol ring and six symmetrically distributed phosphate moieties^[19], is a natural antioxidant in the form of metal chelate that decreases the catalytic reactivity of polyvalent transition metals^[27] and has the anti-carcinogenic effect on colon cancer risk^[19,31]. In addition, resveratrol is a natural phytoalexin in grapes, red wines and other grape products^[32], peanut and its products^[28]. With a resorcin-type structure, resveratrol can inhibit cyclooxygenase-2 (COX-2) activities in colon cancer cells^[33,34] and depress the growth of colorectal aberrant crypt foci^[20], suggesting a protective role in the carcinogenesis of colon cancer.

The association between peanut consumption and human colorectal cancer risk has not been well established. The present study provided cohort data suggesting a significant beneficial effect of peanuts in women. Though legume consumption offers protection against colon cancer^[12], PA and resveratrol are also candidate components in legumes offering this protection. This does not rule out the role of other components such as folic acid, calcium, dietary fiber, protease inhibitors, oligosaccharides, saponins and isoflavones in peanuts and legumes in protecting against colorectal cancer^[35].

Studies on cigarette smoking and colon adenoma rather than colorectal cancer have consistently reported an ele-

vated risk in the earlier twentieth century. However, recent prospective studies showed that smoking is positively related with colorectal cancer, especially in long-term heavy smokers^[36,37]. Giovannuci *et al*^[36] proposed that carcinogens in cigarette smoke might initiate colorectal tumors with an induction period of three to four decades. In our follow-up study, a moderately elevated risk was observed in male smokers with colorectal cancer cases. There is no authentic evidence that can explain why moderate smokers are at the highest risk for colorectal cancer.

We also found that the harmful effect of pickled foodstuff consumption was significantly associated with colorectal cancer in women. Pickled food intake has been considered as a probable risk factor for gastric cancer^[38]. Our finding is in agreement with the result of a case-control study in Japan^[39]. Pickled foodstuff rich in sodium is the main dish for folk vegetarians in Taiwan. However, its association with colorectal cancer has not been found in men, indicating a gender discrepancy.

Increased body mass has been associated with excess colorectal cancer mortality, especially in men^[40]. A previous cases-control study conducted in Taiwan, has also reported a significant gender discrepancy in the risk associated with colorectal cancer^[41]. These findings suggest that the mechanism underlying colorectal carcinogenesis is different between men and women. Gender difference should be considered in studies on the etiology of colorectal cancer.

In conclusion, high bean intake has a number of beneficial effects and high pickled foodstuff consumption may have a number of potentially adverse effects, but the evidence is yet insufficient. Frequent peanut intake can lower the risk for colorectal cancer in women. Intake of peanuts may have beneficial effect on other types of cancer and deserves further study.

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BASIC RESEARCH

Endothelial nitric oxide synthase regulation is altered in pancreas from cirrhotic rats

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INTRODUCTION

Chronic bile duct ligation (BDL) in rats induces severe cirrhosis associated with extra-hepatic organ dysfunction in heart^[1], lung^[2, 3], and extrahepatic vessels such as aorta^[4, 5] and mesenteric artery^[6]. Chronic BDL also induces pulmonary injury associated with hypoxemia and pulmonary vasodilatation due to an excess production of endothelial nitric oxide (eNO)^[2, 3]. In aorta and mesenteric artery from BDL rats, hyporeactivity to vasoconstrictors also originates from NO overproduction^[4-6]. Pancreas may suffer from hemodynamic consequences of cirrhosis, but information on eNOS expression and regulation in this splanchnic organ is missing.

In all types of cirrhotic livers, although eNOS protein expression is increased or decreased, the activity is uniformly decreased^[7-10]. The reason for the decrease in hepatic NO bioavailability remains unclear. One explanation is that caveolin expression, a negative regulatory protein of eNOS, increases during cirrhosis and alters enzyme activity^[10]. However, such negative post-translational regulation of eNOS is not unique.

In isolated endothelial cells, transcriptional and post-transcriptional mechanisms regulating eNOS have been described. Although eNOS activity is associated with changes in Ca²⁺ concentrations within endothelial cells, increase in Ca²⁺ concentration alone is not sufficient to modify enzyme activity^[11]. Binding of calmodulin (CaM) and flow of electrons from the reductase to the oxygenase domains of the enzyme are dependent on phosphorylation and dephosphorylation of various residues of eNOS. Additionally, proteins such as caveolin and heat shock protein 90 (Hsp90) associate with eNOS and modify the enzyme activity as well as its intracellular localization^[12, 13]. eNOS is co-localized with regulatory proteins and other molecules of the signal transduction pathway to form an "eNOS signaling complex" that modulates NO production^[11]. For example, eNOS co-localizes with kinases that phosphorylate eNOS on the ⁴⁹⁵Thr residue with a concomitant decrease in enzyme activity^[11, 14, 15].

The aim of our study was to determine whether biliary cirrhosis could induce pancreatic dysfunction including

Abstract

AIM: To determine whether biliary cirrhosis could induce pancreatic dysfunction such as modifications in endothelial nitric oxide synthase (eNOS) expression and whether the regulation of eNOS could be altered by the regulatory proteins caveolin and heat shock protein 90 (Hsp90), as well as by the modifications of calmodulin binding to eNOS.

METHODS: Immunoprecipitations and Western blotting analysis were performed in pancreas isolated from sham and cirrhotic rats.

RESULTS: Pancreatic injury was minor in cirrhotic rats but eNOS expression importantly decreased with the length (and the severity) of the disease. Because co-immunoprecipitation of eNOS with both Hsp90 and caveolin similarly decreased in cirrhotic rats, eNOS activity was not modified by this mechanism. In contrast, cirrhosis decreased the calmodulin binding to eNOS with a concomitant decrease in eNOS activity.

CONCLUSION: In biliary cirrhosis, pancreatic injury is minor but the pancreatic nitric oxide (NO) production is significantly decreased by two mechanisms: a decreased expression of the enzyme and a decreased binding of calmodulin to eNOS.

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Keywords: Pancreas; Biliary cirrhosis; Endothelial NO synthase; Caveolin; Heat shock protein 90

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modifications in eNOS expression as observed in other extrahepatic organs and modify the regulation of eNOS by regulatory proteins and modifications in eNOS phosphorylation.

MATERIALS AND METHODS

Animals

Sprague-Dawley rats were used in this study and divided into 3 groups, which underwent BDL 15, 30 and 60 days respectively before experiment and were designated as BDL-15, BDL-30 and BDL60 groups respectively.

Induction of biliary cirrhosis

Cirrhosis was induced 15, 30 and 60 days before the experiments. After laparotomy under 2-3% isoflurane anesthesia, Sprague-Dawley rats had a double ligation of the common bile duct with section between the two ligatures. The bile duct was ligated close to the liver to avoid pancreatic duct obstruction. Sham rats had laparotomy without bile duct ligation and were studied 15 days later. Normal rats with no laparotomy served as controls. The protocol was approved by the Animal Welfare Committee of the University of Geneva.

Histologic examination

Tissues were collected from 15 rats ($n=3$ in each group), and 3- μm thick paraffin sections were stained with hematoxylin and eosin as well as the Gomori technique.

Western blotting analysis

Pancreatic tissues were homogenized in lysis buffer containing 50mM Tris HCl, 0.1mM EGTA, 0.1mM EDTA, 1% NP-40, 1% protease inhibitor cocktail, 1% phosphatase inhibitor, cocktails I and II (Sigma). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting analysis were performed using minigels (BioRad, Switzerland). Protein extracts (100 μg) were separated on a 10% (eNOS and hsp90) or a 12% (caveolin and calmodulin) polyacrylamide gel. After the gel was transferred to a polyvinylidene difluoride membrane (Millipore, Volketswil, Switzerland), the membrane was blocked with PBS containing 5% nonfat dry milk (1 h at room temperature) and incubated overnight at 4°C with specific antibodies. The membrane was washed four times with PBS containing 0.2% Tween 20 and incubated for 1 h with an alkaline phosphatase-conjugated second antibody. After washed, protein was detected by chemiluminescence (Immune-Star, Bio-Rad) according to the manufacturer's instructions. Molecular weight markers and positive controls were included for each experiment. Films were scanned using an ImageScanner densitometer equipped with the Labscan and Image Quant software (Amersham Biosciences). An equal quantity of protein stained with Ponceau Red was loaded on each line before the blocking was assessed.

Immunoprecipitation

Immunoprecipitation (IP) was performed by incubating 500 μg or 5 mg of protein extracts with antibodies directed to the native protein eNOS and the immune complexes

were precipitated with protein G-sepharose beads (Sigma, Buchs, Switzerland). Complexes were washed twice in lysis buffer. The samples were boiled in loading buffer (Laemmli sample buffer, BioRad) to elute the bound proteins. Total or phosphorylated eNOS, Hsp90, caveolin and calmodulin were detected by Western blot. Antibodies used included mouse monoclonal anti-eNOS and rabbit polyclonal anti-caveolin-1 from BD Biosciences-Pharmingen (Basel, Switzerland), rat monoclonal anti-Hsp90 from Stressgen (Bioggio, Switzerland), mouse monoclonal anti-calmodulin from Upstate (Lake Placid, New-York) and goat polyclonal anti-phospho-eNOS-Thr⁴⁹⁵ from Santa Cruz Biotechnology (Santa Cruz, California).

Immunostaining

Samples of pancreas ($n=3$ in each group) were fixed in neutral buffered formalin for 24 h, processed and embedded in paraffin. Sections (3- μm thick) were cut and mounted on Superfrost/plus slides, then deparaffinized. The sections were treated with 1mM EDTA (pH=8) to quench endogenous alkaline phosphatase activity. Non-specific binding was blocked by incubating sections in 1% BSA-Tris buffer for 30 min. Sections were then incubated with the primary antibody (rabbit polyclonal anti-caveolin-1 from BD Biosciences-Pharmingen) diluted in BSA-Tris buffer for 1 h. Control sections were incubated in BSA-Tris buffer without the first antibody and showed no immunostaining. After rinsing with Tris, the sections were incubated with alkaline phosphate-labeled polymer (DAKO Envision system) for 10 min and rinsed with Tris between each step. The antigen-antibody complex was visualized using the substrate chromogen solution containing 0.2 mM levamisole for 5-10 min. Sections were subsequently counterstained with Mayer's hematoxylin solution for 2 min and rinsed in distilled water and then in 37 mM ammonia solution (10 times), rinsed in distilled water for 2 min and coverslipped with mounted media glycerol (DAKO). Images were obtained under Zeiss microscope (Zeiss, Zürich, Switzerland) using a Nikon COOLPIX995 camera and processed using the Coll view image and Photoshop software.

A similar technique was used to stain Hsp90 in pancreatic tissues with monoclonal mouse anti-Hsp90 (Stressgen) and eNOS with rabbit polyclonal anti-eNOS (BD Biosciences-Pharmingen). Monoclonal mouse anti-Hsp90 was incubated overnight.

cGMP measurement

Pancreatic cGMP was measured by competitive immunoassay according to the instructions of the manufacturer (R&D systems, Wiesbaden-Nordenstadt, Germany).

Statistical analysis

Data for densitometric measurements were given as mean \pm SD and difference between groups was determined with a Kruskal-Wallis or Mann-Whitney test for non-parametric variables. $P < 0.05$ was considered statistically significant.

RESULTS

Pancreatic injury and biliary cirrhosis

Chronic bile duct ligation induced severe hepatic

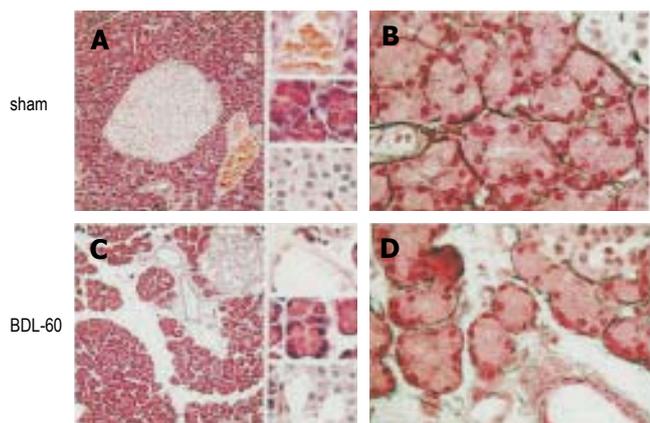


Figure 1 Pancreatic tissues from sham and BDL-60 rats were stained with hematoxylin and eosin (panels A and C) and Gomori (panels B and D). In panels A and C, magnification is 100x and 400x (upper: small vessel; middle: acini; lower: Langerhans islet) and in panels B and D, magnification is 400x. The extracellular matrix was enlarged in pancreas isolated from BDL-60 rats and most of the enlarged extracellular matrix consisted of edema. No increase in reticulin fibers was observed with the Gomori technique. *n* = 3 in each group.

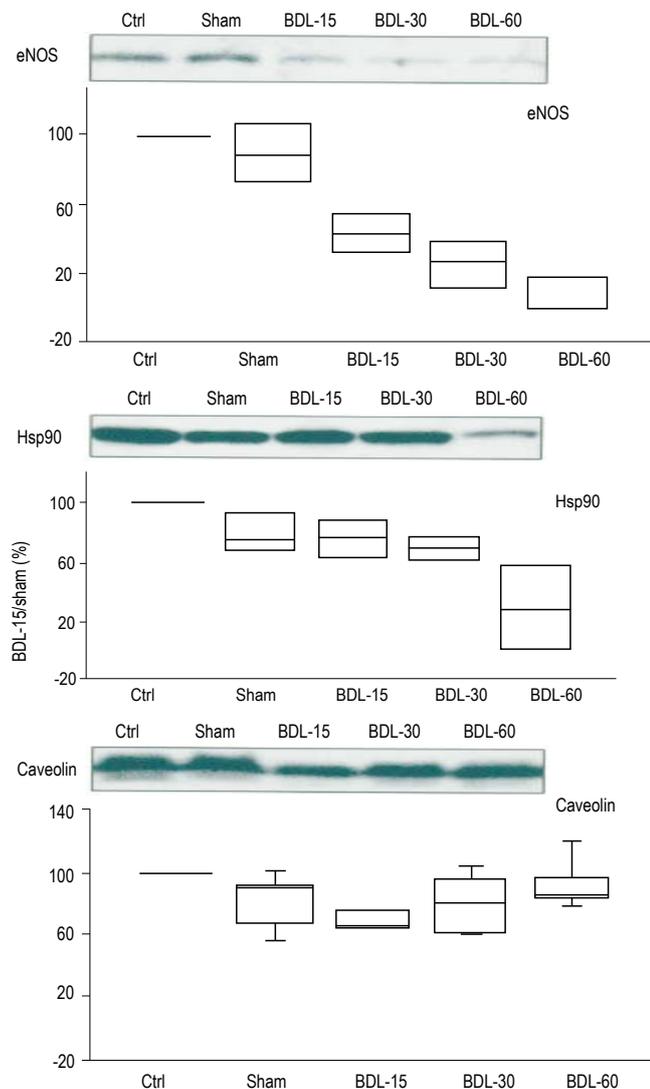


Figure 2 Endothelial nitric oxide synthase (eNOS), heat shock protein 90(Hsp90), and caveolin expression in pancreatic lysates isolated from control (Ctrl), sham rats, and rats with bile duct ligation (BDL) 15, 30, and 60 days before tissue collection (left panel). Densitometry (right panels) was measured in ≥ 4 rats in each group.

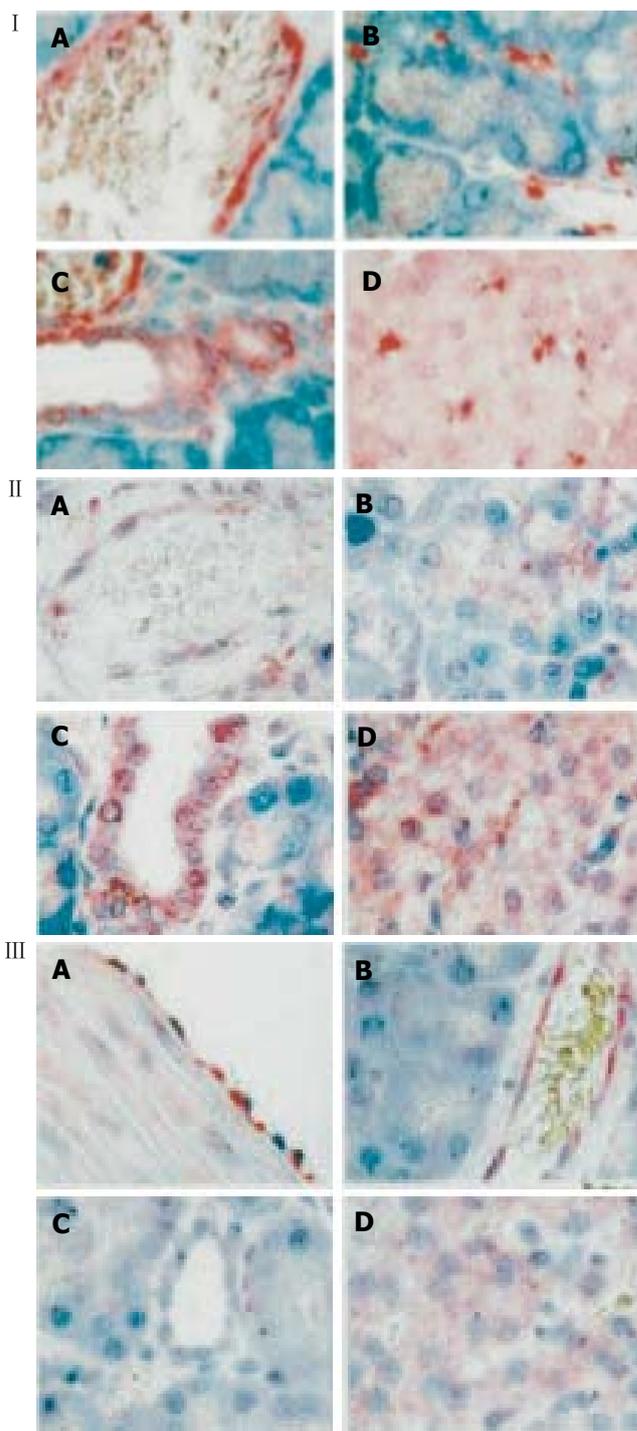


Figure 3 Immunostaining of caveolin (I) in pancreas isolated from BDL-30 rat. Caveolin was detected in epithelial cells lining the pancreatic ductules (C), cells within the islets of Langerhans (D), and in the apex of acinar cells (B). Caveolin was also present in endothelial lining cells in veinules (A), microvessels surrounding acini (B), and microvessels inside Langerhans islets (D). Immunostaining of Hsp90 (II) in pancreas isolated from BDL-15 rat. Hsp90 was present in epithelial cells lining the pancreatic ductules (C), cells within the islets of Langerhans (D), and in the apex of acinar cells (B). Hsp90 was also present in endothelial lining cells in veinules (A). Immunostaining of endothelial nitric oxide synthase (III) in pancreas isolated from BDL-15 rat. The protein was present in endothelial lining cells (B) and cells within the islets of Langerhans (D). In aorta isolated from BDL-60 rats (A), eNOS is more heavily stained. Magnification is 400x.

diseases as evidenced by the increase in serum bilirubin and aspartate aminotransferase concentrations (Table 1). In contrast, serum amylase concentration was

Table 1 Hepatic tests and serum amylase concentrations in sham-operated rats and rats with BDL (mean ± SD)

| | Sham | BDL-15 | BDL-30 | BDL-60 | |
|--------------------------|----------|-----------|------------|------------|------------------|
| ALAT (IU/L) | 34±22 | 60±43 | 76±52 | 56±11 | <i>P</i> =0.55 |
| ASAT (IU/L) | 67±40 | 194±50 | 718±328 | 262±95 | <i>P</i> =0.005 |
| Total bilirubin (μmol/L) | 5.3±6.1 | 154.0±9.2 | 120.7±22.5 | 183.5±28.1 | <i>P</i> <0.0001 |
| Amylase (UI/L) | 1268±113 | 1313±115 | 1439±293 | 2161±299 | <i>P</i> =0.019 |

ALAT = alanine aminotransferase; ASAT = aspartate aminotransferase.

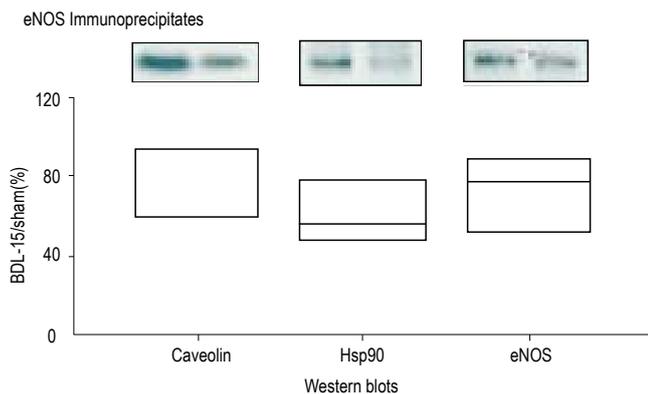


Figure 4 Endothelial NO synthase (eNOS) immunoprecipitation from pancreatic extracts. Immunoprecipitates were Western blotted with Hsp90, caveolin, and eNOS antibodies. Pancreatic proteins were collected from sham rats and rats which had a 15 days bile duct ligation (BDL-15).

slightly increased only in rats with BDL 60 d before the experiment.

Pancreatic injury was minor in rats with BDL. On histological examinations, the only modification was the enlargement of the interstitial space in rats with BDL 60 days before the experiment (BDL-60 rats, Figure 1A).

No pancreatic necrosis was observed at any time point. The enlarged extracellular matrix consisted of edema because reticulin fibers surrounding acini in sham rats did not increase in BDL-60 rats (Figure 1B).

Expression of endothelial NO synthase and its regulatory proteins

eNOS expression was significantly decreased in BDL-15, BDL-30 and BDL-60 rats (*P*=0.003).

The two regulatory proteins of eNOS, Hsp90 (positive regulatory protein of eNOS) and caveolin (negative regulatory protein of eNOS), had different evolution. Hsp90 expression was maintained at the beginning of the disease but significantly decreased in BDL-60 rats (*P*=0.02) while caveolin expression slightly decreased in BDL-15 rats but recovered in BDL-30 and BDL-60 rats (*P*=0.05) (Figure 2).

Protein immunostaining

The localization of caveolin in pancreas is illustrated in Figure 3.

Caveolin was present in epithelial cells lining the pancreatic ductules, cells within the islets of Langerhans

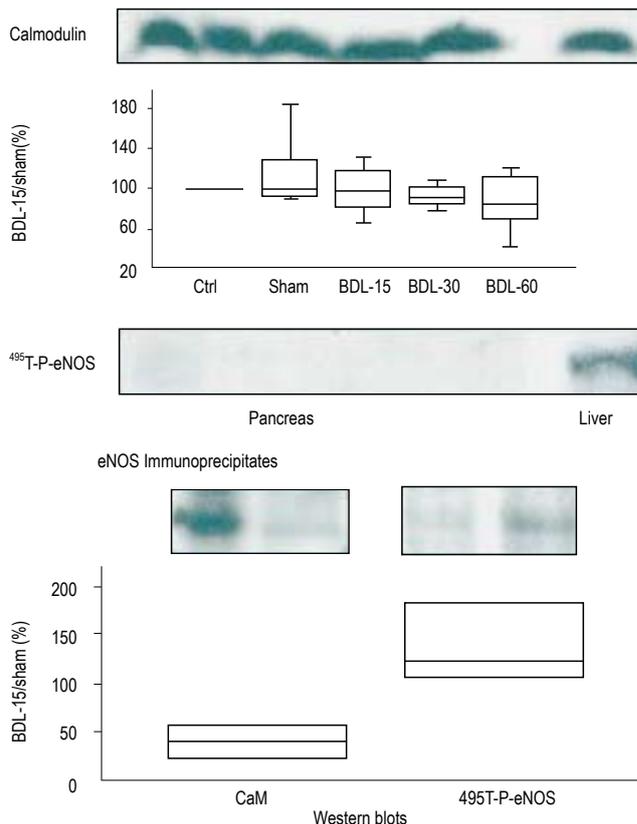


Figure 5 Calmodulin and ⁴⁹⁵Thr-P-eNOS expression in pancreatic lysates isolated from control (Ctrl), sham rats, and rats with bile duct ligation (BDL) 15, 30, and 60 days before tissue collection. Densitometry was measured in ≥ 4 rats in each group. Liver homogenate serves as control.

and in the apex of acinar cells. Caveolin was also present in endothelial lining cells in veinules, microvessels surrounding acini and microvessels within Langerhans islets. Hsp90 was present in epithelial cells lining the pancreatic ductules, cells within the islets of Langerhans and in the apex of acinar cells. Hsp90 was also present in endothelial lining cells in veinules. Immunostaining of eNOS was weak in comparison to caveolin and Hsp90. Endothelial NOS was present only in endothelial cells and cells within the islets of Langerhans. In aorta isolated from BDL-60 rats (used as positive controls), eNOS was more easily detected.

Co-immunoprecipitation of eNOS, caveolin and Hsp90 in sham and BDL-15 rats

Hsp90 and caveolin were co-immunoprecipitated with eNOS in pancreas isolated from sham and BDL-15 rats (Figure 4).

Because in BDL-15 rats, the reduction in coimmunoprecipitation of Hsp90 and caveolin was similar to the decrease in eNOS expression (*P*=0.74), eNOS activity was not modified by this mechanism in BDL-15 rats. Co-immunoprecipitation from pancreas isolated from BDL-30 and BDL-60 rats was not available because the amount of eNOS protein in samples was too low.

Calmodulin and ⁴⁹⁵Thr-P-eNOS expression

In pancreatic lysates, ⁴⁹⁵Thr-P eNOS was not detectable

at any time point, though the phosphorylated eNOS was easily detected in the liver (Figure 5).

Expression of CaM was evident and did not differ between groups ($P=0.95$). In BDL-15 rats, the co-immunoprecipitation of eNOS with CaM decreased while $^{495}\text{Thr-P}$ eNOS became detectable.

Interestingly, the cGMP concentration in pancreatic tissue was slightly lower in BDL-15 rats than in sham rats (62 pmol/g vs 90 pmol/g protein, $P=0.086$).

DISCUSSION

Our study showed that pancreatic injury was mild in rats with chronic biliary cirrhosis. Nevertheless, the expression of eNOS importantly decreased with the duration (and the severity) of the disease. The regulation of eNOS by Hsp90 and caveolin was not modified by a 15-day BDL but cirrhosis decreased the CaM binding to eNOS with a concomitant phosphorylation of the enzyme on ^{495}Thr residue, suggesting that the activity of the enzyme decreases through this regulatory pathway. Consequently, besides liver, lungs and extrahepatic vessels, pancreas is another organ in which eNOS regulation is markedly impaired by cirrhosis.

Chronic biliary cirrhosis

BDL is frequently used to induce chronic biliary cirrhosis in rats. In this model, the severity of hepatic injury increases with the duration of BDL. This model is also characterized by extra-hepatic injuries. Chronic BDL is associated with severe cardiomyopathy^[1]. Vasoconstriction to phenylephrine or vasodilatation to acetylcholine decreases in large vessels such as aorta^[5, 16, 17], mesenteric^[18] and pulmonary arteries^[3]. Additionally, the modifications observed in lungs from BDL rats mimic the hepatopulmonary syndrome that complicates human cirrhosis^[3]. In the present study, we described for the first time a slight pancreatic injury that appeared only 2 months after BDL.

BDL and eNOS expression

Organ dysfunction during biliary cirrhosis includes modification in NO release. eNOS expression has been investigated in livers from normal and BDL rats and the expression is similar in control and BDL-24 rats^[19]. Modifications in eNOS expression have also been found in extrahepatic organs isolated from rats with BDL. For example, in aortic rings isolated from BDL-28 rats, eNOS protein expression is increased. For example, in aortic rings isolated from BDL-28 rats, eNOS protein expression is increased^[20]. These results have been confirmed in rats sacrificed at different time points (BDL-21 and BDL-30 rats)^[5, 21]. The expression of eNOS in pulmonary arteries isolated from BDL-28 rats is also increased^[5, 22]. The high staining of eNOS observed in aorta isolated from BDL-60 rats (Figure 3) might confirm these findings.

In our study, we used the BDL model of cirrhosis. However, additional experimental models exist, including chronic ingestion of CCl₄ that also shows a dysregulation of eNOS^[8]. Though BDL rats develop hepatic cirrhosis with systemic vasodilatation and hepatopulmonary

syndrome as observed in humans^[3], the results obtained in BDL rats should be extrapolated to humans with cautions.

eNOS has already been detected in normal pancreas^[23-25]. For the first time, we showed that eNOS expression significantly decreased in pancreas collected from BDL-15 rats with a further decrease associated with the duration of bile duct ligation. Interestingly, we localized eNOS in endothelial cells and cells within the islets of Langerhans. However, the staining was weak, suggesting that eNOS has a low expression in native pancreas.

eNOS regulation in native tissues

The regulation of eNOS is a process determined by a cascade of events, including changes in (1) NOS mRNA and protein levels, (2) association of eNOS with regulatory proteins in the signaling complex, (3) changes in intracellular location of the enzyme and (4) phosphorylation of serine, threonine and tyrosine residues. Though many of these steps have been relatively well elucidated in *in vitro* models and in cell lines overexpressing one or more components of the signaling complex, much more work is required to determine which modification plays a dominant role in the regulation of eNOS activity in native tissues.

In our experimental study, the two regulatory proteins of eNOS, Hsp90 and caveolin, had a different evolution. Caveolin expression slightly decreased in BDL-15 rats but recovered in BDL-30 and BDL-60 rats. The reason for the transient decrease is unclear. In contrast, in livers from BDL rats, caveolin expression increases^[10]. In normal pancreas, caveolin is detectable in membrane preparations^[26]. Caveolin-1 has been identified in exocrine cells and secretagogues known to stimulate secretions in exocrine cells also release caveolin^[27]. However, the function of secreted caveolin in pancreas is unknown. We also identified caveolin in epithelial cells lining the pancreatic ductules, cells within the islets of Langerhans, endothelial cells lining veinules, microvessels surrounding acini and inside Langerhans islets.

In our study, Hsp90 expression was maintained at the beginning of the disease but significantly decreased in BDL-60 rats. Hsp90 is constitutively expressed at high concentration in normal pancreas and the expression does not increase during acute pancreatitis^[28] or hyperthermia^[29, 30]. In the present study, we showed for the first time, the localization of Hsp90 in epithelial cells lining the pancreatic ductules, cells within the islets of Langerhans, acinar cells and endothelial cells in veinules.

In BDL livers, co-immunoprecipitation of caveolin with eNOS increases and this binding is associated with a decreased eNOS activity^[10]. Co-immunoprecipitation of Hsp90 with eNOS was not investigated in this study. In pancreas from BDL-15 rats, the co-immunoprecipitation of eNOS with both caveolin and Hsp90 similarly decreased, suggesting that the two regulatory proteins have no effect on pancreatic eNOS activity.

Co-immunoprecipitation of eNOS with CaM and eNOS phosphorylation on Thr residue

Phosphorylation of eNOS on the ^{495}Thr residue in isolated endothelial cells is associated with a decrease in enzyme

activity. The link between phosphorylation and NO production can be explained by the interference with the binding of CaM to the CaM-binding domain of eNOS^[11, 14, 15]. Changes in ⁴⁹⁵Thr phosphorylation are generally associated with stimuli such as bradykinin, histamine and Ca²⁺ ionophores. In endothelial cells, these Ca²⁺ elevating agonists decrease ⁴⁹⁵Thr phosphorylation of eNOS with a concomitant increased binding of CaM to the CaM-binding site of eNOS.

Interestingly, we showed a similar regulatory mechanism induced by cirrhosis in native pancreas. The ⁴⁹⁵Thr-phosphorylated form of eNOS undetectable in normal pancreas (in contrast to normal liver) appeared in pancreas from cirrhotic rats. Concomitantly, the increased expression of ⁴⁹⁵Thr-P-eNOS was associated with a decreased binding of CaM to eNOS.

Besides the regulatory proteins and CaM, other proteins are likely to interfere with the regulation of eNOS in the pancreas^[13]. Phosphorylation of ¹¹⁷⁹Ser is another well-identified mechanism activating the enzyme. These regulatory pathways and probably many others concomitantly modify eNOS activity. Although we found a decreased cGMP concentration in pancreas from BDL-15 rats, the numerous pathways regulating eNOS preclude the modifications of eNOS activity to the decreased binding of CaM.

In conclusion, co-immunoprecipitation of Hsp90 and caveolin with eNOS has no effect on eNOS activity while the binding of CaM to eNOS decreases with a likely concomitant decrease in eNOS activity. Besides liver, lungs and extra-hepatic vessels, pancreas is another organ with its eNOS expression modified by cirrhosis.

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BASIC RESEARCH

Erythropoietin -induced proliferation of gastric mucosal cells

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INTRODUCTION

Erythropoietin (EPO) is a glycoprotein (molecular weight = 34 ku) produced in adult kidney and the fetal liver that regulates growth, differentiation, and survival of erythroid progenitors^[1]. The effects of EPO are transduced via EPO receptors (EPO-R), members of the cytokine receptor superfamily, that are primarily expressed on the cell surface of erythroid cells^[2].

Prior to 1990, morbidity and mortality due to hemorrhagic lesions in dialysis patients was relatively high^[3,4]. However, the impact of these complications has been attenuated by the advent of EPO which is used for treatment of renal anemia and by the marked reduction in the incidence of peptic ulcer disease due to the eradication of *H pylori*. Studies on gastric mucosal defense factors have focused on the protective role of gastric mucosal blood flow, oxygen supply, and radical oxygen suppression, and the importance of ferrous ions in the gastric mucosa has also been demonstrated^[5,6]. However, direct effects of EPO on the gastric mucosa have not fully been investigated.

Recently, the effect of recombinant human erythropoietin (rhEPO) on cellular proliferation has been examined in human umbilical vein endothelial cells (HUVEC)^[7] and bovine pulmonary artery endothelial cells^[8]. The growth of these cells was stimulated by EPO. Further, we have previously demonstrated the growth promoting effects of EPO on porcine gastric mucosal vascular endothelial cell^[9]. These effects may improve the gastric lesions found in dialysis patients through the neovascularization in mucosal tissue.

Okada *et al*^[10] reported that EPO stimulated proliferation of rat gastric-derived cell lines via EPO-R expressed on these cells. Although these rat stomach

Abstract

AIM: To analyze the localization of erythropoietin receptor on gastric specimens and characterize the effects of erythropoietin on the normal gastric epithelial proliferation using a porcine gastric epithelial cell culture model.

METHODS: Erythropoietin receptor was detected by RT-PCR, Western blotting and immunohistochemistry. Growth stimulation effects of erythropoietin on cultured gastric mucosal cells were determined by ELISA using bromodeoxyuridine (BrdU).

RESULTS: Erythropoietin receptor was detected on cultured porcine gastric mucosal epithelial cells. Erythropoietin receptor was also detected histochemically at the base of gastric mucosal epithelium. BrdU assay demonstrated a dose-dependent increase in growth potential of cultured porcine gastric mucosal epithelial cells by administration of erythropoietin, as well as these effects were inhibited by administration of anti-erythropoietin antibody ($P < 0.01$).

CONCLUSION: These findings indicate that erythropoietin has a potential to proliferate gastric mucosal epithelium via erythropoietin receptor.

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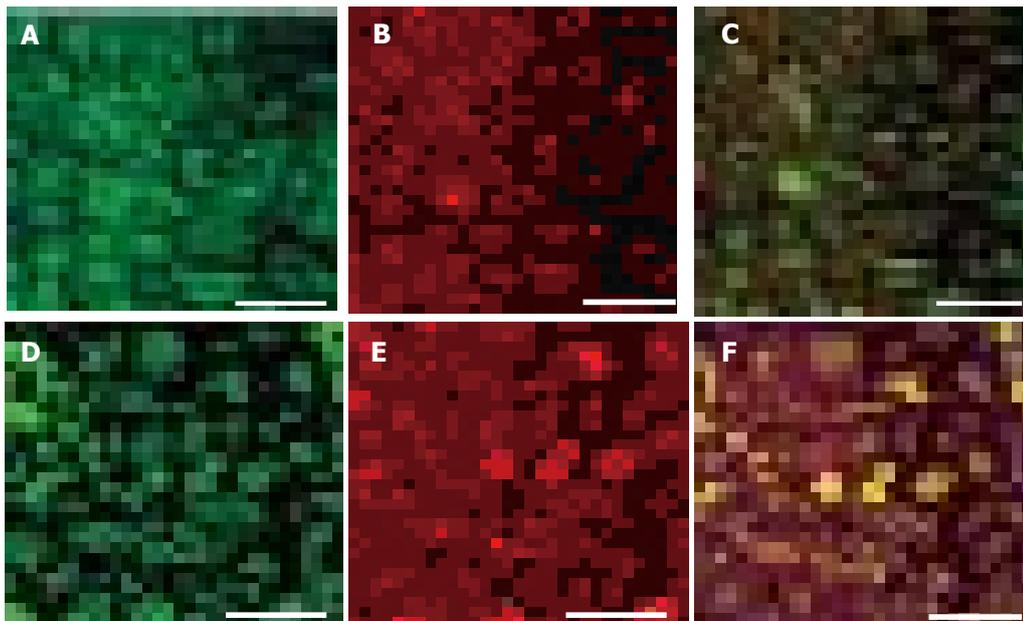


Figure 1 Expression of pepsin, MUC5AC and proton-pump in cultured gastric epithelial cells observed by double immunolabelling. A confocal microscopic image demonstrates the FITC-positive anti-pepsin-positive cells (A) and Texas Red-positive anti-MUC5AC-positive cells (B) in the same area. The anti-pepsin-positive cells, which co-express MUC5AC, are indicated yellow (C). A confocal microscopic image also demonstrates the FITC-positive anti-pepsin-positive cells (D) and Texas Red-positive anti-proton pump-positive cells (E) in the same area. The anti-pepsin-positive cells which co-express proton pump are indicated yellow (F). Scale bars indicate 100 μm .

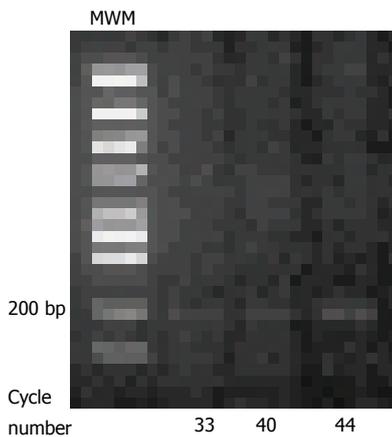


Figure 2 RT-PCR analysis of EPO-R in cultured gastric epithelial cells. 207 bp products are detected. MWM; molecular weight marker.

derived cells were morphologically epithelioid, they were also immortalized with very short doubling times, which suggests that these cells had undergone transformation and possess characteristics that differ from normal gastric epithelium. Indeed, many types of tumor cells express EPO-R and show increased growth in response to EPO exposure *in vitro*^[11]. Therefore, the goal of the present study was to analyze the localization of EPO-R on gastric epithelium and to characterize the effects of EPO on the normal gastric epithelium using a porcine gastric epithelial cell culture model.

MATERIALS AND METHODS

Reagents

Collagenase and EDTA were purchased from Wako Pure Chemicals (Tokyo, Japan). Keratinocyte-SFM medium, modified Eagle medium (MEM), Dulbecco's modified minimum essential medium, trypsin, newborn calf serum, TRIzol reagent and penicillin-streptomycin mixture were purchased from Gibco BRL (Gland Island, NY). Human

recombinant basic fibroblast growth factor was purchased from Intergen (Purchase, NY). rhEPO (Epoetin Beta) was purchased from Chugai Pharmaceutical Co. (Tokyo, Japan). Monoclonal anti-porcine H⁺/K⁺ ATPase antibody was purchased from Affinity Bioreagents (Golden, CO). Anti-porcine stomach pepsin goat antibody was purchased from Polysciences (Warrington, PA). Monoclonal anti-human gastric mucin antibody (MUC5AC) was purchased from NeoMarkers (Union City, CA). Rabbit anti-human EPO-R antibody was kindly provided by collaborative researcher: Dr. M. Higuchi of Chugai Pharmaceutical Co. (Tokyo, Japan). Isotype matched rabbit IgG against anti-EPO antibody was from Chemicon (Temecula, CA). Horseradish peroxidase-labeled rabbit IgG antibody was purchased from Zymed Laboratories Inc. (South San Francisco, CA). Texas Red labeled anti-mouse IgG and FITC-labeled anti-goat IgG were purchased from Vector Laboratories (Burlingame, CA).

Cell culture

Porcine gastric mucosal epithelial cells were separated by the method with modification which has been developed for the cultivation of human pulmonary epithelial cells^[12]. Fetal porcine gastric mucosa was digested with 0.1% collagenase, and cells obtained were seeded onto plastic dishes in plain medium (Dulbecco's minimum essential medium supplemented with 10% newborn calf serum, 100 U/mL of penicillin and 100 $\mu\text{g}/\text{mL}$ of streptomycin) and incubated at 37 °C. The viability of the separated cells was about 80%–90%. After the epithelial cell colonies grew large enough, other cells were detached from the dish manually with a small piece of silicon rubber under the phase contrast microscopy. The colonies were collected with small grass cylinder (internal diameter 10 mm) and 0.05% trypsin-0.02% EDTA, and the cells were seeded onto new dishes using the growth medium (keratinocyte-SFM containing 10% new born calf serum and 10 ng/mL of basic fibroblast growth factor). The culture medium was changed twice a week. The cells were passaged in the

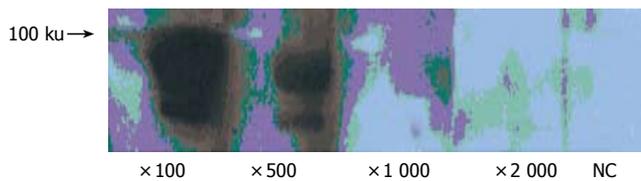


Figure 3 Western blotting analysis of EPO-R in cultured gastric epithelial cells. EPO-R is detected as a predicted size. The intensity of signals is dependent on anti-EPO-R antibody dilution.

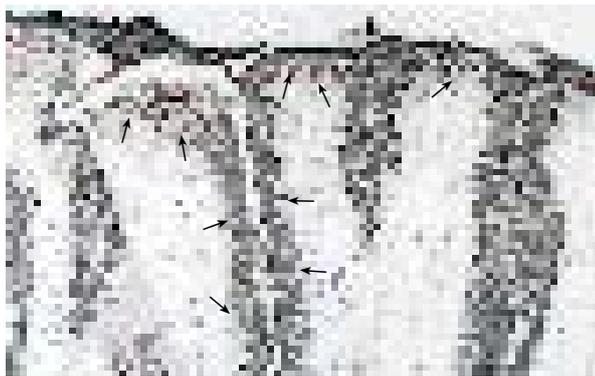


Figure 4 Immunolocalization of EPO-R and MUC5AC on porcine gastric mucosa. Immunolocalization of EPO-R as well as gastric mucin MUC5AC is detected in epithelial cells. ($\times 60$).

split at the ratio 1:4 and were used in younger generation (passages 6-7).

Identification of gastric mucosal epithelial cells in culture

Cells were also identified as gastric epithelial cells by double staining with anti-porcine H⁺/K⁺ ATPase antibody, anti-pepsin antibody and anti-gastric mucin antibody in order to identify for gastric parietal cells, chief cells and mucin-producing cells, respectively. Cell growth assessed by the overnight bromodeoxyuridine (BrdU) uptake showed that about 60% of the cells retained growth potential and the population doubling time was about 36 h in these culture conditions.

EPO-R gene expression on gastric mucosal epithelial cells

Analysis of EPO-R by RT-PCR: EPO-R gene expression in porcine gastric mucosal epithelial cells (1×10^7) was analyzed by RT-PCR. Total RNA was extracted using TRIzol reagent. RNA samples were treated with RNase-free DNase I at 37°C for 30 min to eliminate genomic DNA contamination. RNA was purified with 1 volume of phenol:chloroform (5:1). RT-PCR was performed by RNA PCR Kit (AMV) ver. 2.1 (Takara, Japan). Briefly, 1 μ g of RNA was primed with random primers and reverse transcribed using reverse transcriptase in a final volume 20 μ L. 5 μ L of this mixture was PCR-amplified in a 25 μ L reaction using Takara Taq DNA polymerase. The following PCR priming sets were used under PCR conditions of 94°C for 45 s, 58°C for 1 min, and 72°C for 2 min. EPO-R: forward primer (5'-AGC TGT GGC TGT ACC AAA CTG A-3'), reverse primer (5'-ACT TGT

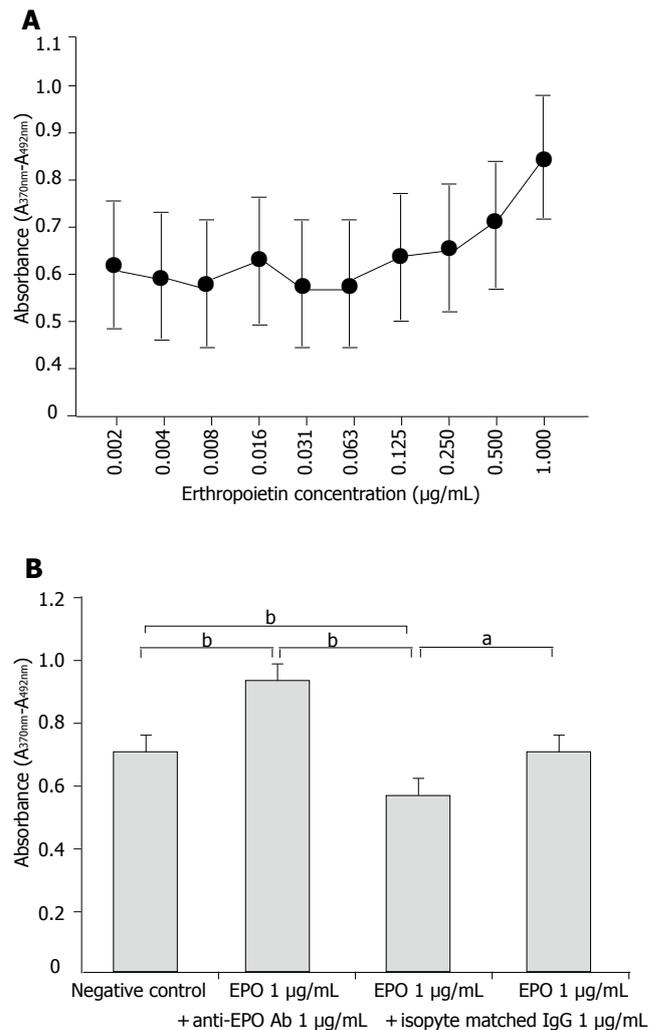


Figure 5 Evaluation of erythropoietin effects on cultured gastric epithelial cells. (A) BrdU assay shows a dose-dependent effect of EPO on growth stimulation of porcine gastric mucosal epithelial cells. (B) Growth stimulation effects of EPO on gastric mucosal epithelial cells. Treatment with EPO (closed bar) significantly increased cell growth. This effect is inhibited by anti-EPO neutralizing antibody (hatched bar), not by isotype matched IgG (dotted bar). Results are expressed as the means \pm SD of six different experiments. ^a $P < 0.05$, ^b $P < 0.01$.

CCA GCA CCA GGT AGG T-3'); number of cycles: 36, 40, and 44. All PCR products were separated on ethidium bromide-stained gel.

Analysis of EPO-R by Western blotting

Porcine epithelial cells ($0.5 - 1 \times 10^7$) were cultured in modified Eagle's medium (MEM) containing 10% fetal calf serum (FCS) for 48 h. EPO (1 μ g/mL) was added, and the cells were incubated for an additional 24 h. The cells were homogenized in cold buffer. The protein concentration was measured by DC protein assay kit (Bio-Rad, CA) according to the manufacturer's instruction. Aliquots of 50 μ g protein were applied each lane of 10% SDS-polyacrylamide gel and electrophoresed. After electrophoresis, samples were transferred onto PVDF membrane (Millipore, MA). The filter was then incubated with anti-human EPO-R monoclonal antibody (diluted 1:100, 1:500, 1:1000 and 1:2000 respectively). Reaction product was demonstrated using ECL-plus Kit (Amersham).

Bioscience, UK). For a negative control experiment, the primary antibody was omitted from the solution.

Analysis of EPO-R in the tissues by Immunohistochemical staining

Stomachs were resected from pig fetuses and dissected along the greater curvature. The gastric mucosa was visually inspected, removed from the anterior wall, fixed in 10% formalin, embedded in paraffin, and cut into 5 μ m sections. Primary antibodies against EPO-R and MUC5AC were used in the immunohistochemical staining. Tissue sections were incubated overnight with primary antibodies at 4°C and then treated with horse radish peroxidase-labeled rabbit IgG antibody for 1hr at room temperature. The immunoreactive products were visualized with DAB staining (ABC method). Counterstaining was performed with hematoxylin. For negative controls, the primary antibody was replaced with rabbit or mouse nonimmune serum. Double staining for EPO-R and MUC5AC was performed with DAB staining (ABC method) followed by Ni-DAB staining (PAP method). For negative controls, the protocol was similar to that used for single staining.

Growth stimulation effects of EPO on cultured porcine gastric mucosal epithelial cells

Cultured porcine gastric mucosal epithelial cells were incubated with Keratinocyte-SFM containing 20% newborn calf serum. EPO (0.002, 0.004, 0.008, 0.016, 0.031, 0.063, 0.125, 0.25, 0.5, 1 μ g/mL) was added to the supernatant to evaluate the dose-dependent effects on growth potential of the gastric mucosal cells.

To evaluate the specificity of EPO effects, four sets of groups were established: 1) untreated cultures; 2) EPO 1 μ g/mL treated cultures; 3) EPO 1 μ g/mL + anti-EPO antibody 1 μ g/mL treated cultures; and 4) EPO 1 μ g/mL + nonspecific antibody (rabbit IgG) 1 μ g/mL treated cultures. Quantification of cell growth was measured by ELISA using BrdU.

Cultures and quantitative analysis were performed using 96-well plates (Nunc, Naperville, IL). Medium was prepared for each experimental group and aliquoted (50 μ L/well) into 6 wells per group. The gastric mucosal epithelial cells were adjusted to a density of 1×10^5 cells/mL, and 50 μ L were aliquoted into each well and incubated (5000 cells/well) for 4 d. In accordance with a cell proliferation ELISA protocol, 10 μ L/well of BrdU solution was added, and incubation was continued for 24 h. After discarding the supernatants, 200 μ L/well of fixation reagent was added for 30 min to fix the cells and denature the DNA. After discarding the supernatants, 100 μ L/well of anti-BrdU peroxidase solution was added and followed by incubation for 1 h. After discarding the supernatants and washing, 100 μ L/well of substrate solution was added and incubated for 30 min, and absorbance at 370 nm (control wavelength, 492 nm) was assessed.

Statistical analysis

All results are expressed as mean \pm SE. Statistical comparisons were made using the Student *t*-test or Welch's *t*-test after the analysis of variances. The results were considered significantly different at $P < 0.05$.

RESULTS

Identification of gastric mucosal epithelial cells in culture

Cells were identified as gastric epithelial cells by double staining with anti-proton pump antibody, anti-pepsin antibody and anti-gastric mucin antibody in order to identify for gastric parietal cells, chief cells and mucin-producing cells, respectively (Figure 1). In the most cells, immunofluorescence showed double positive staining for anti-pepsin antibody and anti-proton pump antibody or for anti-pepsin antibody and anti-gastric mucin antibody.

EPO-R gene expression on gastric mucosal epithelial cells

RT-PCR: EPO-R was detected on the cultured porcine gastric mucosal epithelial cells. 207 bp products were detected (Figure 2).

Western blotting

We examined the expression of EPO-R protein in porcine epithelial cells by Western blotting. EPO-R protein expression was detected as a predicated size. The intensity of signals was dependent on anti-EPO-R antibody dilution (Figure 3).

Immunohistochemical staining of EPO-R in the gastric tissue

EPO-R showed a punctate expression pattern at the base of gastric mucosal epithelial cells. MUC5AC was observed in the mucosal cell reticulum. Double staining was performed to examine the correlation between mucosal epithelial expression of EPO-R and presence of glycoproteins, which are an important component of the mucosal barrier. The findings were positive for EPO-R in the foveolar epithelial cells that were also positive for MUC5AC (Figure 4)

Growth stimulation effects of EPO on gastric mucosal epithelial cells

BrdU assay demonstrated a dose-dependent increase in growth potential of cultured porcine gastric mucosal epithelial cells at EPO doses ranging from 0.002 to 1 μ g/mL. Evaluation of EPO dose dependence in this study showed the greatest growth potential at 1 μ g/mL (Figure 5A) compared to untreated cultures, cultures treated with EPO showed statistically significant growth stimulation. In contrast, co-culturing with EPO antibody showed a significant growth inhibition effect. On the other hand, there was no significant inhibition effect of cell growth with a nonspecific antibody. These findings suggested that growth stimulation by EPO of gastric mucosal cells was mediated via EPO-specific receptors (Figure 5B).

DISCUSSION

The incidence of gastrointestinal bleeding is higher in hemodialysis patients than in the general population. The advent and use of histamine-2 (H_2) receptor blockers and proton pump inhibitors (PPIs) has resulted in a small decrease in gastrointestinal bleeding events in hemodialysis

patients, but the use of EPO therapy for treatment of anemia associated with renal failure has led to a more dramatic decrease in this complication for reasons that were previously unclear. Decreased blood supply secondary to anemia leads to low local oxygen concentrations in the gastric mucosa and results in formation of gastric lesions in dialysis patients^[3,4,6]. Thus an increase in red blood cell production in response to EPO may reduce the incidence of hemorrhagic lesions by increasing oxygen delivery to the gastric mucosa. However, the present study suggests that the direct effect of EPO to gastric mucosa may also mediate the efficacy of EPO therapy found in dialysis patients.

The EPO gene was cloned and recombinant human EPO was purified in 1985, followed by identification of EPO-R on erythroid cells using iodine-labeled EPO. The EPO-R density on the surface of erythroid cells is low, ranging from a few hundred to a few thousand per cell. In the hematopoietic system, EPO-R is mainly expressed at the stage of late erythroid progenitor cells,^[13] and the number of receptors decreases with differentiation into mature erythrocytes. In addition to erythroid progenitors, EPO-R is expressed on megakaryocytes^[14], vascular smooth muscle cells^[15], endothelial cells^[7] skeletal myoblasts^[16], neural cells^[17], renal cells^[18], ischemic retinal cells^[19] and in the epicardium and pericardium^[20]. Further, studies of the digestive tract have shown EPO-R mRNA in hepatic stromal cells in fetal mice^[21], and EPO-R protein has been found in the intestinal villi during development in humans and rats.^[22,23] Okada *et al*^[10] investigated whether EPO stimulated gastric mucosal development *in vitro* in a rat gastric mucosal cell line (RGM-1) and reported that EPO produced a dose-dependent increase in thymidine uptake and c-myc gene expression. In addition, RT-PCR confirmed EPO-R gene expression, thus showing a direct growth effect on RGM-1 cells. It should be noted, however, that RGM-1 may represent transformed cell line rather than normal gastric epithelial cells. We previously demonstrated EPO-R mRNA expression in cultured porcine gastric mucosal microvascular endothelial cells^[9].

In the present study, we confirmed the EPO-R mRNA in the cultured normal gastric epithelial cells. In addition, we used immunohistochemical analysis in the tissue sections to demonstrate EPO-R immunoreactivity in gastric mucosa cell, predominantly in foveolar epithelial cells. These data suggests that EPO may play a direct role in modulating normal gastric mucosal cells.

The dose-dependent effects of EPO on mucosal epithelial cell growth were observed at doses up to 1 µg/mL. Treatment of the porcine gastric mucosal epithelial cells with EPO clearly resulted in increased cell growth. The increase was inhibited by co-culturing with anti-EPO antibody, with significantly lower growth than in cells co-cultured with nonspecific antibody (rabbit IgG), which suggests that the growth stimulation effect of EPO is a direct and specific effect on the gastric mucosal epithelial cells.

EPO may have direct action on other types of mucosal and stromal cells. A previous study reported that treatment of rats with EPO before surgical anastomosis increased anastomosis strength and improved healing^[24]. Further,

in a retrospective study in premature rats, treatment with EPO decreased the incidence of experimentally-induced necrotizing enteritis^[25,26]. In addition, treatment with EPO, both orally and parenterally, increased jejunal villus surface area. As compared to a control group, villus length was increased in groups treated with oral or parenteral EPO^[27]. These findings indicate that EPO has a trophic action on the small intestine. EPO also stimulates growth of hepatic stromal cells in fetal mice, and furthermore, there is a dose-dependent effect. These results are consistent with data from the present study performed in gastric mucosal cells. EPO-specific mucosal cell growth is a noteworthy finding in our study, and the present study suggests EPO may prevent hemorrhagic gastric lesions in dialysis patients by a systemic hematopoietic effect and by a local effect on the gastric mucosa. EPO directly and specifically stimulated gastric mucosal epithelial cell proliferation.

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BASIC RESEARCH

Effect of hepatic iron concentration reduction on hepatic fibrosis and damage in rats with cholestatic liver disease

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INTRODUCTION

Chronic liver injury due to various causes may result in hepatic fibrosis, which can lead to cirrhosis and end-stage liver failure^[1]. Currently, there are no proven therapeutic options designed to delay or reverse the progression of hepatic fibrosis. Iron overload is a relatively common finding in many patients with a variety of end-stage liver diseases, who have no evidence of genetic hemochromatosis^[2, 3]. In such patients the presence of hepatic siderosis is associated with more advanced liver dysfunction, more rapid decompensation and decreased survival^[4].

The pathogenesis of hepatic fibrosis in patients with iron overload is not well understood. Four possible mechanisms have been suggested. (1) Iron *per se* is as an inducer of fibrosis even in the absence of necrosis and inflammation, and may act as a profibrogenic agent that stimulates the deposition of collagen by hepatic stellate cells^[5]. (2) Iron, as a mediator of hepatocellular necrosis and local inflammation, may activate the peroxidative process, produce oxygen-free radicals, lipid peroxidation damage to protein and DNA and stimulation of fibrosis^[6-11]. (3) Iron, as an inducer of fibrosis, may act in conjunction with other hepatotoxins^[6]. It has been suggested that mild to moderate hepatic iron concentration (HIC) increase in patients and animals with various liver diseases, like non-alcoholic steatohepatitis, hepatitis C virus (HCV) infection and alcoholic liver disease, may exacerbate liver injury and accelerate the development of hepatic fibrosis^[12-20]. Moreover in HCV positive patients undergoing liver transplantation, HIC increase in the donor liver (graft) is associated with fibrosis progression^[21]. (4) Iron overload may alter hepatic extracellular matrix degradation^[22]. In patients with hemochromatosis, the ratio between serum concentration of matrix metalloproteinases (MMP's) 1-3 and tissue inhibitor of metalloproteinase 1 is reduced as compared to controls. Iron reduction therapy causes increase in MMP's 2 and 3 serum concentration^[22].

In the liver, iron is present in a few forms: ferritin,

Abstract

AIM: To assess the effect of iron reduction after phlebotomy in rats with "normal" hepatic iron concentration (HIC) on the progression of hepatic fibrosis, as a result of bile duct ligation (BDL).

METHODS: Rats underwent phlebotomy before or after sham operation or BDL. Animals undergone only BDL or sham operation served as controls. Two weeks after surgery, indices of hepatic damage and fibrosis were evaluated.

RESULTS: Phlebotomy lowered HIC. Phlebotomy after BDL was associated with body weight increase, lower hepatic weight, less portal hypertension, less periportal necrosis, less portal inflammation, lower hepatic activity index score and higher albumin levels. On the other hand, phlebotomy before BDL was associated with body weight decrease and hepatic activity index score increase. Phlebotomy after sham operation was not associated with any hepatic or systemic adverse effects.

CONCLUSION: Reduction of HIC after induction of liver damage may have beneficial effects in BDL rats. However, iron deficiency could induce impairment of liver function and may make the liver more susceptible to insults like BDL.

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Key words: Iron; Phlebotomy; Bile duct ligation; Hepatic activity index; Rat.

Table 1 Weights and phlebotomy volume in different groups of rats (mean \pm SE)

| Subgroup | <i>n</i> | Weight gain (% from baseline) | Mean phlebotomy Volume ¹ (mL) | Liver weight (% of total body weight) | Splenic weight(% of total body weight) |
|---------------------------|----------|----------------------------------|---|--|--|
| Sham only | 5 | 15.03 \pm 18.70 ^a | 0 \pm 0 | 3.97 \pm 0.16 ^a | 0.41 \pm 0.07 ^a |
| Sham and phlebotomy later | 4 | 9.4 \pm 7.0 | 0.70 \pm 0.13 | 3.62 \pm 0.32 | 0.37 \pm 0.04 |
| Phlebotomy and sham later | 6 | -5.7 \pm 17.3 | 0.86 \pm 0.18 | 3.47 \pm 0.19 | 0.38 \pm 0.03 |
| BDL only | 9 | -4.99 \pm 3.90 | 0 \pm 0 | 6.89 \pm 1.29 | 0.66 \pm 0.10 |
| BDL and phlebotomy later | 8 | 8.01 \pm 3.1 ^a | 0.71 \pm 0.16 | 5.97 \pm 0.45 | 0.56 \pm 0.07 |
| Phlebotomy and BDL later | 8 | -3.29 \pm 20.1 | 0.80 \pm 0.15 | 7.02 \pm 1.68 | 0.69 \pm 0.13 |

¹Mean phlebotomy volume for one phlebotomy session per rat; ^a*P* \leq 0.01 vs BDL only.

Table 2 Liver function tests and enzymes in different groups of rats (mean \pm SE)

| Subgroup | <i>n</i> | GGT (IU/L) | ALK- P (IU/L) | Bilirubin (μ mol/L) | GPT (IU/L) | Albumin (G/L) |
|---------------------------|----------|----------------------------|---------------------------|-----------------------------|--------------------------|-----------------------------|
| Sham only | 5 | 7.0 \pm 2.0 ¹ | 254 \pm 32 ¹ | 4.6 \pm 1.8 ¹ | 53 \pm 11 ² | 28.0 \pm 1.1 ¹ |
| Sham and phlebotomy later | 4 | 6.0 \pm 1.4 | 305 \pm 58 | 4.0 \pm 0.8 | 66 \pm 8 | 29.2 \pm 2.2 |
| Phlebotomy and sham later | 6 | 14.3 \pm 10.3 | 305 \pm 61 | 41.7 \pm 39.0 | 55 \pm 20 | 28.4 \pm 2.3 |
| BDL only | 9 | 74.0 \pm 63.4 | 580 \pm 174 | 106.0 \pm 57.0 | 115 \pm 50 | 24.3 \pm 2.2 |
| BDL and phlebotomy later | 8 | 65.9 \pm 17.9 | 578 \pm 75 | 104.0 \pm 16.2 | 125 \pm 22 | 29.0 \pm 2.0 ¹ |
| Phlebotomy and BDL later | 8 | 64.3 \pm 17.6 | 665 \pm 166 | 109.0 \pm 39.0 | 144 \pm 40 | 23.7 \pm 2.1 |

¹*P* \leq 0.007, ²*P* \leq 0.02 vs BDL only GGT=Gamma-glutamyl transpeptidase, ALK-P=Alkaline phosphatase, GPT=Alanine aminotransferase. No difference in the levels of serum parameters like calcium, phosphor, urea, creatinine and electrolytes were found between the different groups (data not shown).

hemosiderin, heme and as low molecular weight iron (LMWI). It is thought that part of LMWI bound to weak chelators like ATP and AMP, is readily available to induce tissue damage^[23]. This fraction of iron is probably the most susceptible to reduction after iron reduction therapy.

Iron depletion has been reported to improve metabolic derangement in patients with non-alcoholic fatty liver disease, liver damage and response to drug therapy in patients with chronic HCV infection^[13, 24-27]. Menstruating women with chronic HCV infection who are iron deficient have lower aminotransferase levels and improved liver histology when compared to age-matched menstruating females who are not iron deficient^[28]. The possibility of altering the natural history and clinical course of HCV infection and to improve the response to anti-viral therapy (interferon monotherapy) has led to the recommendation to consider iron reduction therapy (phlebotomy) in patients with HCV infection as an adjuvant therapy to the other therapeutic modalities, even if HIC is normal or only mildly elevated^[29,30]. Plebotomy is safe in most HCV-infected patients who are usually not iron deficient^[31]. In HCV-infected patients in whom ribavirin is combined

Table 3 Histological grading and staging and bile duct proliferation score in different groups of rats (mean \pm SE)

| Subgroup | <i>n</i> | Periportal necrosis | Portal inflammation | Lobular necrosis | Fibrosis | Bile duct proliferation |
|---------------------------|----------|---------------------|---------------------|------------------|------------------------------|------------------------------|
| Sham only | 5 | 0.00 \pm 0.00 | 0.40 \pm 0.55 | 1.00 \pm 1.22 | 0.00 \pm 0.00 ¹ | 0.00 \pm 0.00 ¹ |
| Sham and phlebotomy later | 4 | 0.00 \pm 0.00 | 1.25 \pm 1.26 | 0.25 \pm 0.50 | 0.00 \pm 0.00 | 0.00 \pm 0.00 |
| Phlebotomy and sham later | 6 | 0.00 \pm 0.00 | 1.00 \pm 0.00 | 0.50 \pm 0.55 | 0.67 \pm 0.52 | 0.00 \pm 0.00 |
| BDL only | 9 | 0.89 \pm 1.27 | 1.33 \pm 1.00 | 2.11 \pm 1.83 | 2.89 \pm 0.78 | 2.67 \pm 0.50 |
| BDL and phlebotomy later | 8 | 0.13 \pm 0.35 | 1.00 \pm 0.00 | 3.00 \pm 1.85 | 2.75 \pm 0.71 | 2.88 \pm 0.35 |
| Phlebotomy and BDL later | 8 | 2.17 \pm 1.33 | 2.67 \pm 0.82 | 4.00 \pm 0.00 | 3.33 \pm 0.52 | 2.83 \pm 0.41 |

¹*P* \leq 0.003 vs BDL only.

with interferon, HIC does not influence the response to therapy^[32]. However, it is not yet known whether phlebotomy may improve the sustained virological response or histological score in these patients. Patients with chronic cholestatic liver disease do not usually have hepatic iron overload^[4]. Moreover, no information exists as to the effect of iron reduction in subjects with cholestatic liver diseases with no increase ("normal levels") of HIC.

The aim of the present work was to assess the effect of iron reduction after phlebotomy on the HIC in rats with "normal" iron stores and to evaluate whether reduction of HIC may decrease the hepatic damage and slow the progression of hepatic fibrosis induced by bile duct ligation (BDL).

MATERIALS AND METHODS

Forty male Sprague Dawley rats (Harlan, Israel), weighing 180-223 g, were studied. Rats were housed in regular cages situated in an animal room at 24°C with a 12-h light-dark cycle. Rats were maintained on standard rat chow (Kofflok, Tel Aviv, Israel) and with free access to tap water. The Ethics Review Committee for Animal Experimentation of the Hebrew University Hadassah Medical School approved all the animal studies described

Table 4 Hepatic prostaglandin E₂ (PGE₂) generation, myeloperoxidase (MPO) activity and hydroxyproline (HP) content in different groups of rats (mean ± SE)

| Subgroup | n | PGE ₂ generation (ng/g liver) | HP content (ng/g liver) | MPO activity (units/g liver) |
|---------------------------|---|--|-------------------------|------------------------------|
| Sham only | 5 | 14.8±9.9 | 8.59±0.55 ¹ | 0.45±0.14 |
| Sham and phlebotomy later | 4 | 14.7±5.2 | 8.11±1.03 | 0.31±0.12 |
| Phlebotomy and sham later | 6 | 6.9±3.2 | 8.02±1.00 | 0.18±0.05 |
| BDL only | 9 | 8.5±4.5 | 11.00±0.97 | 0.59±0.21 |
| BDL and phlebotomy later | 8 | 3.9±2.1 ¹ | 9.80±1.68 | 0.52±0.10 |
| Phlebotomy and BDL later | 8 | 3.4±2.3 ¹ | 11.90±1.40 | 0.27±0.17 ¹ |

¹P≤0.018 vs BDL only.

herein. BDL was performed as previously described^[33]. Under ketamine anesthesia (100 mg/kg, intramuscular) a median laparotomy was done, the common bile duct was exposed, doubly ligated with 3/0 silk and sectioned between ligatures. The abdominal cavity was closed with two suture lines. In sham operated (SO) rats the portal area and liver hilum were exposed, the common bile duct was isolated and gently mobilized.

Three groups of animals were studied: (1) Control group of BDL (n=9) and SO (n=5) rats; (2) Post-procedure phlebotomy group of BDL (n=8) and SO (n=4) rats. These rats were phlebotomized on a daily basis for five consecutive days, from the fourth to the ninth post-operative days. Phlebotomy was done through a tail vein with 21-gauge needles under light ether anesthesia; (3) Pre-procedure phlebotomy group of BDL (n=8) and SO (n=6) rats. These rats were phlebotomized on a daily basis for five consecutive days from the ninth to the fourth days before the surgical procedure (BDL or sham operation).

Two weeks after the surgical procedure rats were anesthetized with ether. The abdominal cavity was opened by midline incision, and blood was drawn from the inferior vena cava, centrifuged, aliquoted and frozen. Serum albumin, bilirubin, calcium, phosphor, liver enzymes, kidney function tests and electrolytes were measured by the dry chemistry method (Kodak, Rochester, NY, USA). Serum iron (SI) as determined by Guanidine/FerroZine method (Cobas Integra 700). Transferin was determined using the immunoturbidimetric method (Cobas Integra 700). The liver and spleen were removed and weighed. Sections from the liver of each animal included in the study were taken for histological evaluation, determination of HIC, myeloperoxidase (MPO) activity, prostaglandin E₂ (PGE₂) generation and hydroxyproline (HP) content.

Liver histology

Sections from the liver of each animal were fixed in phosphate-buffered formaldehyde, embedded in paraffin, and 5-μm thick sections were prepared. Sections were stained with hematoxylin and eosin for evaluation of necroinflammatory grading and Masson's trichrome stains for fibrosis and architectural changes. Histological

Table 5 Hepatic iron content and concentration in different groups of rats (mean ± SE)

| Subgroup | n | Iron concentration (μg/g/liver) | Iron content (μg/total liver) |
|---------------------------|---|---------------------------------|-------------------------------|
| Sham only | 5 | 91.8±12.9 | 861.3±158.9 |
| Sham and phlebotomy later | 4 | 40.1±8.8 ³ | 315.1±63.3 ³ |
| Phlebotomy and sham later | 6 | 55.0±19.7 | 358.9±104.4 |
| BDL only | 9 | 80.9±17.2 | 1122.1±358.6 |
| BDL and phlebotomy later | 8 | 37.4±6.9 ¹ | 477.2±85.1 ¹ |
| Phlebotomy and BDL later | 8 | 49.0±29.6 | 628.0±224.7 ² |

¹P≤0.0001, ²P≤0.012 vs BDL only; ³P≤0.016 vs sham only.

grading of BDL-induced liver damage was assessed by a modification of the Knodell score^[34]. Each specimen was examined for the following features and scored by points of increment from 0-4, according to the severity of the findings as follows: periportal necrosis: none = 0, mild = 1, moderate = 2, marked = 3, severe = 4; portal inflammation: none = 0, mild = 1, moderate = 2, marked = 3, severe = 4; lobular necrosis: none = 0, mild = 1, moderate = 2, marked = 3, severe = 4; fibrosis: none = 0, portal expansion = 1, septal formation = 2, marked bridging fibrosis = 3, cirrhosis = 4. Hepatic activity index (HAI) was calculated for each rat by summation of the points of these four parameters. In addition, bile duct proliferation was assessed in each specimen by allocating points of increment from 0-4, according to the severity of the findings. Histological evaluation of the pathological specimens was done on a blind basis by our pathologist. Reliable correlation was seen in repeated and blinded histological evaluation of the specimens.

Determination of PGE₂

One hundred and fifty mg of liver tissue was placed in preweighed tubes containing 1.0 mL of phosphate buffer (50 mmol/L, pH 7.4). The liver was minced with scissors and centrifuged in an Eppendorf centrifuge for 10 s. The pellet was resuspended in 1.0 mL of the above buffer, incubated for 1 min in a vortex mixer. Indomethacin (10 μg) was added and the tubes were centrifuged for 60 s. The supernatant was kept at -20°C until radioimmuno-assay was performed. The ability of liver tissue to generate PGE₂ was expressed as ng/g wet tissue weight. PGE₂ generation was determined by radioimmunoassay as previously described^[33].

Determination of MPO activity

Liver tissue (200 mg) was homogenized three times for 30 s each time at 4°C with a polytron (Kinematica GmbH, Krietz-Lucern, Switzerland) in 1.0 mL of ice-cold 0.5% hexadecyltrimethylammonium bromide in 50 mmol/L phosphate buffer, pH 6.0. The polytron probe was rinsed twice with 1.0 mL of the buffer and the washing was added to the homogenate. The homogenate was then sonicated for 10 s, freeze-thawed three times and centrifuged for 15 min at 40 000 r/min. An aliquot of the supernatant was

Table 6 Serum iron and transferrin in different groups of rats (mean \pm SE)

| Subgroup | n | Serum iron (μ g/dL) | Transferrin (mg/dL) |
|---------------------------|---|--------------------------|---------------------|
| Sham only | 5 | 163 \pm 21 | 446 \pm 46 |
| Sham and phlebotomy later | 4 | 144 \pm 38 | 459 \pm 17 |
| Phlebotomy and sham later | 6 | 160 \pm 20 | 482 \pm 103 |
| BDL only | 9 | 205 \pm 71 | 640 \pm 128 |
| BDL and phlebotomy later | 8 | 197 \pm 57 | 732 \pm 65 |
| Phlebotomy and BDL later | 8 | 215 \pm 116 | 701 \pm 83 |

taken for determination of enzyme activity according to Bradley *et al.*^[35]. The correlation coefficient based on 10 standard curves was $r=0.98$ and inter assay variation was $0 \pm 1.65\%$ (mean \pm SE, $n=57$).

Determination of hepatic iron content

Hepatic non-heme iron concentration was measured by the method of Torrance and Bothwell^[36].

Determination of hepatic hydroxyproline content

Hepatic hydroxyproline content was measured by the method described by Woessner^[37].

Statistical analysis

Data were expressed as mean \pm SE. Group variance was analyzed by the Kruskal-Wallis test, followed by the Mann-Whitney test for multiple comparisons to allow pair-wise testing for significant differences between groups. $P \leq 0.05$ was considered statistically significant.

RESULTS

Effect of bile duct ligation

Two weeks after BDL all rats were jaundiced, had lesser weight gain, greater liver and spleen weight than SO rats (Table 1). BDL rats also had higher serum levels of liver enzymes, higher bilirubin and lower albumin levels, higher fibrosis score, higher hepatic HP content, higher hepatic activity index and higher bile duct proliferation score than SO rats (Table 2, Tables 3 and 4 and Figure 1). No change in HIC and content or SI and transferrin were observed as a result of BDL (Tables 5 and 6).

Effect of phlebotomy

Phlebotomy after surgical procedure (SO or BDL) resulted in a decrease of HIC and content. The change was more pronounced in BDL rats than in SO rats. Rats that were phlebotomized before the surgical procedure had higher HIC than those undergone phlebotomy after the surgical procedure (Table 5). No change in SI and transferrin was noted in any group as a result of phlebotomy (Table 6).

Phlebotomy after BDL was associated with body weight in crease, higher albumin levels and lower hepatic PGE₂ generation at sacrifice compared to BDL only rats (Tables 1-3). Moreover, periportal necrosis, portal inflammation, fibrosis scores, total HAI and hepatic HP content decreased in rats that were phlebotomized after BDL, although the results did not reach statistical significance. Phlebotomy before BDL was associated

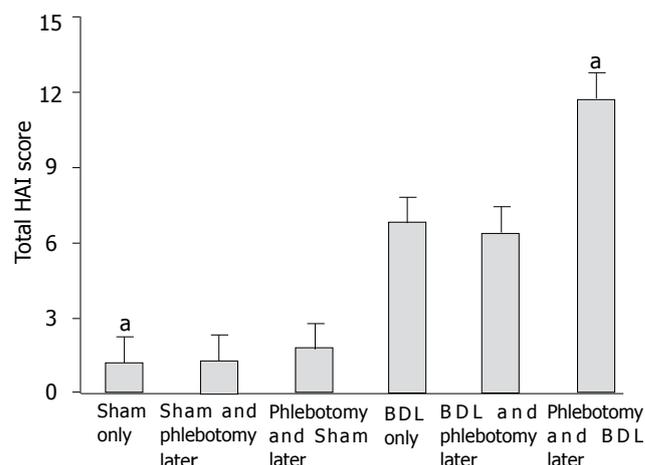


Figure 1 Hepatic activity index (HAI) in the study groups; ^a $P < 0.02$ vs BDL only.

(when compared to BDL only rats) with a similar weight loss. Periportal necrosis, portal inflammatory activity and fibrosis score were higher in this group than in the BDL only group. However, while the difference in value for each variable was not statistically significant, the combination of variables (HAI) was significant (12.17 ± 1.93 vs 7.22 ± 3.49 , $P \leq 0.018$, Figure 1). Surprisingly, hepatic PGE₂ generation and MPO activity in the rats that had phlebotomy before BDL were lower than those in the BDL only group.

When both phlebotomy groups undergone BDL were compared, the group that had phlebotomy after the procedure had a better outcome: weight gain, higher albumin level, lower hepatic weight ($P=0.029$), less portal hypertension (determined as lower spleen weight) ($P=0.029$), less periportal necrosis ($P=0.008$) and portal inflammation ($P=0.008$) and lower total HAI score ($P=0.008$, Tables 1-3, Figure 1).

DISCUSSION

The role of iron in the progression of hepatic damage in various clinical and experimental conditions has usually been studied by iron loading^[14,16,18-20], or by iron depletion in situations where mild to moderate iron overload is present^[13,24,25,30]. In the present study, we examined the effect of reduction of "normal" hepatic iron stores on liver function and histology in rats subjected to BDL. This situation may be more relevant and analogous to and may mimic more closely the common clinical conditions where most of the patients with non-hemochromatosis and non-end-stage liver diseases do not have iron overload^[13].

In the present study we were able to demonstrate that it was possible to reduce HIC by phlebotomy and that such a procedure after sham operation was not associated with hepatic adverse events. Liver disease developed in the rats after two weeks of BDL was not associated with HIC increase. Phlebotomy before the surgical procedure was associated with HIC reduction but not with SI or transferrin reduction. The changes in liver histology (mainly necrosis and inflammation) following the HIC reduction indicated that hepatic iron might not have a role in the fibrosis process after BDL^[38]. Alternatively, we may also speculate that further reduction of HIC by more

aggressive phlebotomy would have an effect on fibrosis. Since SI and transferrin levels did not change following phlebotomy, we assume that the phlebotomy process performed in our rats was not sufficient to alter the course of the fibrosis process. Although no change was observed in fibrosis score, phlebotomized rats after BDL exhibited favorable effects. These rats had body weight increase, higher albumin levels and lower hepatic necroinflammatory activity. Iron deficiency induced by iron chelation may reduce the severity of inflammation in joints and in the gastrointestinal tract^[39, 40].

HIC was only mildly reduced in the rats undergone phlebotomy before the surgical procedure compared to the BDL only rats. Only the total amount of iron per whole liver was reduced significantly. This could be due to the ability of rats to absorb iron during the time interval between the last phlebotomy session and the sacrifice of the rats (18 d). This may indicate that phlebotomy has a transient effect and that continuous phlebotomy sessions over time should be done in order to sustain a beneficial effect. In cirrhotic subjects an increased iron absorption due to increased duodenal expression of iron transporter, divalent metal transporter 1 has been reported^[41]. However this phenomenon does not exist in rats with cholestatic liver disease.

Phlebotomy had a favorable effect on the rats after BDL, but a definite negative effect before BDL. The latter rats lost weight and had a worse HAI (a worse score in periportal necrosis and portal inflammation) as compared to the other BDL groups. A similar trend of changes was observed in the rats subjected to phlebotomy before sham operation. Iron deficiency may affect various organs including eccentric cardiac hypertrophy in rats^[42] and various hepatic enzyme functions^[43]. Anemic patients with early cirrhosis may have worse hemodynamic parameters (lower systemic vascular resistance, increased cardiac index and hepatic venous pressure gradient) than non-anemic control patients^[44]. Increased blood hemoglobin may attenuate splanchnic vasodilatation in portal hypertensive subjects by nitric oxide inactivation^[44]. In our study, the rats undergone phlebotomy before BDL had the highest splenic weight (a marker of portal hypertension). Based on this finding we may speculate that deterioration of hemodynamic parameters in these rats is associated with activation of the renin-angiotensin system. This system may have a role in hepatic fibrosis in BDL rats^[45].

Hepatic PGE₂ generation and MPO activity were used as adjuvant markers for hepatic inflammatory activity in our study, and decreased PGE₂ generation was observed in the BDL groups undergone phlebotomy. This is unexpected as iron is known to down-regulate PGE₂ generation in various tissues^[46, 47]. Higher PGE₂ levels were also expected in the BDL groups with increased hepatic inflammatory changes. This is similar to the results in our previous study of rats to BDL and colitis, in which colonic PGE₂ generation was reduced as compared with a control group^[33]. One may speculate that cyclooxygenase activity or even phospholipase A₂ activity may be defective in rats with experimental liver disease^[33, 48].

As MPO is found primarily in neutrophil granules, it is a marker of neutrophil content and influx into tissues^[49]. Our previous study in BDL rats with colitis

found that mucosal inflammation is correlated with MPO activity^[33]. In the present study, however no correlation was found between the hepatic inflammatory changes and hepatic MPO activity. Decreased MPO activity in the Carrageenan model of inflammation in rats with acute cholestasis due to bile duct resection has been reported^[50]. Additional defects of neutrophil function in BDL rats, like impairment of adhesion, defective phagocytosis, increased superoxide generation and chemotaxis, and increased number of neutrophil counts, have been reported^[50-53]. Iron deficiency *per se*, may also impair neutrophil function and MPO activity and cause a selective defect in the process of inflammation^[54, 55]. Iron repletion improves some of the above functions^[54]. In this study, hepatic MPO activity was the lowest in the rats undergone phlebotomy before the surgical procedure. However there was no clear correlation between HIC, hepatic MPO activity and hepatic inflammation.

In summary, hepatic iron depletion in rats after a liver insult like BDL, may have beneficial effects. "Hepatic iron deficiency" may make the liver more vulnerable to insults such as laparotomy or BDL. Hepatic PGE₂ generation and hepatic MPO activity are not reliable markers for hepatic inflammation in rats with cholestatic liver disease.

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BASIC RESEARCH

Contrast-enhanced endoscopic ultrasound in discrimination between focal pancreatitis and pancreatic cancer

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CONCLUSION: Contrast-enhanced endoscopic ultrasound improves the differentiation between chronic pancreatitis and pancreatic carcinoma.

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Key words: Endoscopic ultrasound; Contrast enhancer; Chronic pancreatitis; Pancreatic cancer

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Abstract

AIM: To evaluate the contrast-enhanced endosonography as a method of differentiating inflammation from pancreatic carcinoma based on perfusion characteristics of microvessels.

METHODS: In 86 patients with suspected chronic pancreatitis (age: 62 ± 12 years; sex: f/m 38/48), pancreatic lesions were examined by conventional endoscopic B-mode, power Doppler ultrasound and contrast-enhanced power mode (Hitachi EUB 525, SonoVue[®], 2.4 mL, Bracco) using the following criteria for malignant lesions: no detectable vascularisation using conventional power Doppler scanning, irregular appearance of arterial vessels over a short distance using SonoVue[®] contrast-enhanced technique and no detectable venous vessels inside the lesion. A malignant lesion was assumed if all criteria were detectable [gold standard endoscopic ultrasound (EUS)-guided fine needle aspiration cytology, operation]. The criteria of chronic pancreatitis without neoplasia were defined as no detectable vascularisation before injection of SonoVue[®], regular appearance of vessels over a distance of at least 20 mm after injection of SonoVue[®] and detection of arterial and venous vessels.

RESULTS: The sensitivity and specificity of conventional EUS were 73.2% and 83.3% respectively for pancreatic cancer. The sensitivity of contrast-enhanced EUS increased to 91.1% in 51 of 56 patients with malignant pancreatic lesion and the specificity increased to 93.3% in 28 of 30 patients with chronic inflammatory pancreatic disease.

INTRODUCTION

Endoscopic ultrasound is a diagnostic method with a high sensitivity for detection of small pancreatic lesions and can rule out carcinomas in almost all patients without chronic pancreatitis^[1,2]. However, the differential diagnosis of pancreatic lesions, especially malignant neoplasia in patients with chronic pancreatitis is always difficult.

Differentiation between malignant and benign lesions in the liver is possible in many cases through analysis of its organ specific vascularity (portal vein/sinusoidal) using liver specific contrast media^[3,4]. In contrast to the liver with its organ specific (portal vein/sinusoidal) blood supply, the pancreas does not contain pancreatic specific vasculature. Therefore, the differentiation of malignant from benign pancreatic lesions seems to remain an unsolved problem today though technology is improved. The same problem arises using computerized tomography (CT) and magnetic resonance imaging (MRI)^[5-7]. In patients with chronic pancreatitis the specificity of differentiation between malignant and benign lesions in the pancreas is reported to be as low as 33%-75%^[8,9].

However, the introduction of endoscopic ultrasound-guided fine needle aspiration has made this easier^[10-12]. The gold standard is still surgery in combination with pathological examination of histological specimens. Nevertheless an improved non-invasive method of detecting malignant tissue is preferable. In the present study, contrast-enhanced endoscopic ultrasound was used to differentiate between benign and malignant pancreatic lesions.

MATERIALS AND METHODS

Patients

A total of 120 patients (age: 61 ± 18 years; sex: f/m 56/64) who showed undifferentiated pancreatic lesions in ultrasound and computed tomography were included into the present study. Reference imaging examinations (e.g. computed tomography, magnetic resonance imaging and positron emission tomography [PET]) were performed as part of the clinical work-up of the patients. Patients with mucinous cyst adenoma ($n=1$), serous cyst adenoma ($n=5$), serous cyst adenocarcinoma ($n=4$) and neuroendocrine tumours ($n=6$) were excluded from the study. Contrast-enhanced endoscopic ultrasound was not performed in seven patients due to severe heart failure NYHA III/IV, a contraindication for SonoVue[®]. The image quality was inadequate in one patient due to severe pancreatic lipomatosis. Furthermore, the lesion could not be reached in 10 patients by the biopsy needle. Finally, 86 patients (age: 62 ± 12 years; sex: f/m 38/48) were included in the study.

Informed consent according to the ethical guidelines from Helsinki was obtained from all patients after the patients were informed of the purpose of the study before ultrasound examination was started.

Methods

Endoscopic ultrasound was performed using an electronic linear ultrasound probe (Pentax FG 38 UX; Hitachi EUB 525). The pancreas was examined as previously described^[13]. As contrast enhancing agent, SonoVue[®] (Bracco, Milano, Italy) was applied in all patients. During the procedure all patients were monitored as previously described^[14,15].

Conventional B-mode and power Doppler scanning

The imaging criteria of chronic pancreatitis have been recently discussed^[16,17]. All patients received transabdominal examination as previously described^[18]. In this study the specificity and sensitivity of endoscopic ultrasound were tested before and after injection of the contrast-enhancing agent SonoVue[®] in differentiating focal chronic pancreatitis from pancreatic carcinoma. Tumour location, morphology, echogenicity and size were documented. If a lesion was detected, vascularity and possible vessel infiltration were evaluated using conventional endoscopic power Doppler ultrasonography in comparison to the surrounding pancreatic tissues and regarded as hyper-, iso- or hypovascular.

The pulse repetition frequency and wall filters were individually adjusted to enable the detection of parenchymal and intratumoral vessels and to avoid artefacts such as "blooming". To detect vascular complications such as portal vein thrombosis, the portal vein was also investigated in variable scans. If any echogenic material was found in the lumen of the portal vein, power Doppler was used to detect flow inside the material. If flow was detected, pulsed wave Doppler analysis was performed to differentiate between pulsatile arterial and non-pulsatile (portal-) venous perfusion. In addition, the following criteria for malignant lesions were used: irregular lesion without clear structure, stenosis of pancreatic duct with prestenotic duct dilatation

and infiltration of vessels or surrounding organs.

Contrast-enhanced power Doppler scanning

In a prestudy, Levovist and SonoVue[®] were compared in 10 consecutive patients, which demonstrated best results in imaging pancreatic perfusion using SonoVue (2.4 mL). Therefore, contrast-enhanced ultrasound was applied using SonoVue[®] 2.4 mL (BR1, Bracco, Italy) to evaluate if this contrast application could improve the characterisation of tumour vascularity. SonoVue[®] (2.4 mL) was injected following a flash of 10 mL saline solution via a catheter (12 mm in diameter or larger) into a cubital vein. Hitachi EUB 525 was used. Continuous scanning was performed with its pulse repetition frequency set at 7 kHz to gain optimal signals as low as possible but to avoid artefacts with an adjusted wall filter. In contrast to the technique described by Becker *et al.*^[13], we combined the analysis of the detected vessels with pulse wave (pw) Doppler analysis. The sonograms were stored on videotapes and re-evaluated blind to the clinical settings using a semiquantitative scoring system to evaluate the vascularity during different phases of perfusion.

Criteria of chronic pancreatitis without neoplasia were defined as no detectable vascularisation or regular appearance of vessels over a distance of at least 20 mm before and after injection of SonoVue[®] and detection of arterial and venous vessels in the contrast-enhanced phase.

Criteria for malignancy were no detectable vascularisation before injection of SonoVue[®], irregular appearance of arterial vessels over a short distance after injection of SonoVue[®] and no detection of venous vessels in the lesion. A malignant lesion was assumed if all criteria were detectable.

The whole procedures were stored on videotapes. The criteria of benign and malignant pancreatic tumour using contrast-enhanced power Doppler are summarized in (Table 1)

Endoscopy-guided puncture

EUS-guided fine needle aspiration was performed in all patients. The patients with benign diseases were followed up for at least 12 mo. After detection of the lesion by endoscopic ultrasound, the diagnostic criteria of endoscopic ultrasound were used to get a primary diagnosis. Then the lesion was investigated with the help of power Doppler mode before and after the application of 24 mL SonoVue[®] for over 3 min and the secondary diagnosis was established. At the end of the procedure all lesions were punctured with a 22 G fine needle aspiration system (Cook Deutschland GmbH, Mönchengladbach, Deutschland) to confirm the diagnosis using cytology. For optimization of the cytology results more than 6 needle passes of the lesion were used as previously described^[10,19]. The material was spread out on to a normal microscopic glass platelet and dried by air. Then, a blood stain (May-Gruenwald/Giemsa) was made. In case of remaining tissue, the tissue biopsy was fixed in formalin for histological examination. A malignant process was assumed if the result of cytological examination was Papanicolaou IV or V or the histological examination was positive for malignant tissue. A benign process was assumed if the result of

Table 1 Endosonographic criteria for differentiation between malignant and benign diseases

| Kind of lesion | Vessel architecture using conventional power mode | Arterial or venous vessels in the lesion using continuous duplex scanning | Vessel architecture using contrast-enhanced power mode |
|--------------------|---|---|--|
| Pancreatic cancer | No visible vascularisation | Only arterial vessels | Irregular, chaotic vessels |
| Focal pancreatitis | Nearly no visible vascularisation | Both arterial and venous vessels in the lesion | Regular with no changing diameter |

Table 2 Endoscopic findings in B-mode ultrasound in patients with pancreatic carcinoma and focal chronic pancreatitis

| | | Pancreatic carcinoma | Chronic pancreatitis |
|-------------------------|--------------|----------------------|----------------------|
| Good quality of imaging | | 56 | 30 |
| Localization | Head | 35 | 25 |
| | Corpus | 12 | 4 |
| | Tail | 9 | 1 |
| Size (cm -SD) | | 3.8 (1.09) | 3.02 (0.94) |
| Echogenicity | Hypoechoic | 41 | 3 |
| | Mixed echoic | 15 | 27 |
| Borders | Clear | 15 | 25 |
| | Irregular | 41 | 5 |
| Structure | Irregular | 41 | 5 |
| | Regular | 15 | 25 |
| Ducts | Dilated | 39 | 6 |
| | Not dilated | 17 | 24 |
| Lymphnodes | Visible | 15 | 4 |
| | Not present | 41 | 26 |
| Invasion | Visible | 23 | 0 |
| | Not visible | 33 | 30 |

cytological examination was Papanicolaou I or II.

Final diagnostic methodology

After detection of the lesion by endoscopic ultrasound and the use of contrast-enhanced Doppler sonography, the final diagnosis was achieved by endoscopy-guided puncture. The material was immediately evaluated and re-puncture was performed if the material was not representative. Re-puncture was successful in 17 patients. Surgery was performed in 19 patients leading to concordant results. There was an inoperable tumour in the following diagnostic procedures (for example metastasis of lung, condition of the patient etc.) or no other sign of pancreatic cancer in all other patients. If the operation was refused, we re-examined the patients with assumed chronic pancreatitis 6 and 12 mo after the diagnosis. In case of no signs of malignant tumour 12 mo after the initial diagnosis, pancreatic cancer was ruled out.

RESULTS

Final diagnosis

Final diagnosis was obtained by endoscopy-guided puncture in all 86 patients. Furthermore the diagnosis was confirmed by surgery in 19 patients and by re-examination after 6 and 12 mo in all other patients with suspect chronic pancreatitis.

Conventional B-mode and power Doppler scanning

Adequate visualisation of the pancreas and the suspected lesion could be achieved in all patients. The diagnosis was

confirmed by conventional B-mode and power Doppler endoscopic ultrasound in 41 of 56 patients using more than 2 of the previously described 3 B-mode ultrasound criteria for malignant lesions. The sensitivity was 73.2%. The diagnosis was confirmed in absence of the mentioned criteria in 25 of 30 patients with focal chronic pancreatitis. The specificity was 83.3%. However, these results were comparable to other reports^[8]. The findings are shown in (Table 2).

Contrast-enhanced power Doppler scanning

The main criteria for malignant lesions were the complete absence of venous vessels by using power Doppler in combination with pw-Doppler examination of the lesion within 3 min after contrast enhancer application. The quality of imaging was acceptable if the time was long enough to scan all visible vessels by pw-Doppler. In this study, application of the contrast enhancer SonoVue had to be repeated in 4 patients to achieve adequate image quality. In addition, using the criteria of Becker *et al*^[13], hypervascularity was observed in the area of interest in 26 patients and hypovascularity in 4 patients with chronic pancreatitis (specificity 86.6%). Hypervascularity was observed in 10 patients and hypoperfusion in 46 patients pancreatic carcinoma (sensitivity 82.1%).

The diagnosis was confirmed in 51 of 56 patients with pancreatic carcinoma using the main criteria of malignant vascularisation with a sensitivity of 91.1%. The diagnosis was confirmed in 28 of 30 patients with focal chronic pancreatitis using the criteria of benign vascularisation with a specificity of 93.3%. The results are shown in Figure 1. Typical endoscopic ultrasound images after injection of contrast enhancer SonoVue[®] in power Doppler mode are demonstrated in Figures 2 and 3.

DISCUSSION

Contrast-enhanced techniques provide information on vascularity and blood flow in normal and pathological tissues. The use of 2nd generation contrast agents in combination with low mechanical index techniques can improve the transabdominal application of contrast-enhanced techniques allowing real-time imaging with or without three dimensional reconstruction. In contrast to the frequently applied transabdominal technique^[20,21] there are only a few reports on contrast-enhanced endoscopic ultrasound^[13,22-24]. Endoscopic ultrasound has been widely used in the past 20 years while real time contrast using low mechanical index (MI) is still restricted to the transabdominal application. In the present study, we showed that contrast-enhanced power Doppler ultrasonography in combination with power Doppler analysis could improve the differentiation between malignancy and chronic pancreatitis.

The pressure inside ductal adenocarcinoma of the pan-

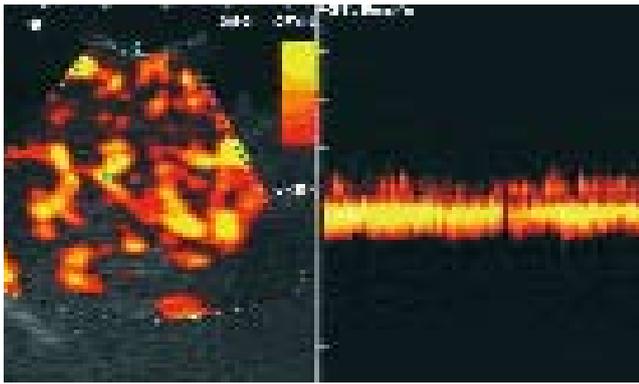


Figure 1 Endoscopic ultrasound image of chronic pancreatitis. Regular vascularisation is shown with detection of venous vessels using contrast-enhanced power Doppler scanning in combination with power Doppler.

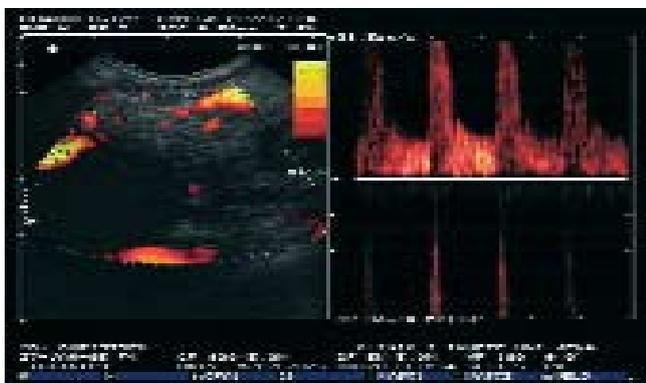


Figure 2 Endoscopic ultrasound image of ductal adenocarcinoma of the pancreas. Irregular vascularisation is shown with detection of only arterial and no venous vessels using contrast-enhanced power Doppler scanning in combination with pw-Doppler.

creas is higher due to surrounding fibrous tissues, which could explain the observed phenomenon that only arterial signals can be displayed in patients with malignancy with contrast-enhanced Doppler techniques, whereas both arterial and venous signals can be displayed in patients with chronic pancreatitis.

We rarely observed detectable vascularity and irregular vessel architecture in almost all patients with pancreatic carcinoma after application of the contrast-enhancing agent SonoVue®. However, both venous and arterial vessels were detectable in benign lesions. In contrast, only arterial vessels were observed in malignant lesions. The sensitivity in the discrimination between benign and malignant pancreatic lesions increased from 73.2% by endoscopic ultrasound to 91.1% using contrast-enhanced power Doppler ultrasound combination with power Doppler endoscopic ultrasound. In addition, the specificity increased from 83.3% to 93.3%. The analysis of contrast enhancing vessels in the pancreatic masses by power Doppler ultrasound in combination with power-Doppler endoscopic ultrasound is superior to the analysis of the intensity of vascularisation by power Doppler ultrasound only as demonstrated by Becker *et al*^[13].

The different vessel structures in malignant tissue are in accordance with a recently published study analysing the

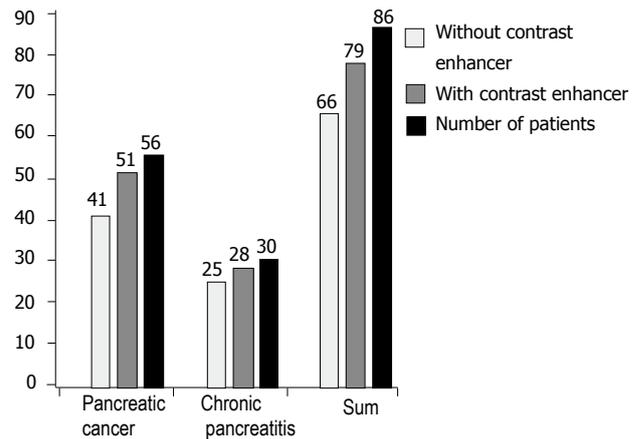


Figure 3 Results of contrast-enhanced endoscopic ultrasound in differentiation between pancreatic carcinoma and focal pancreatitis.

architecture of vessels in pancreatic carcinoma in rats^[25]. However, Weskott *et al*^[26] reported that venous vessels are rarely found in malignant lymph nodes.

Bhutani *et al*^[9] have observed an effect of Levovist® imaging on abdominal vessels in pigs. In 1998, Hirooka *et al*^[22] demonstrated that lesions in gallbladder and pancreas can be visualized using the contrast enhancer Albunex®, focal pancreatitis and pancreatic carcinoma can be differentiated by analysing the contrast enhancement. They also reported that patients with focal chronic pancreatitis reveal enhancement whereas patients with pancreatic carcinoma have no enhancement^[25].

Recently, Nomura *et al*^[24] showed that improved staging of oesophageal and gastric cancer after application of an ultrasound contrast enhancer leads to better visualisation of the gut layers. Up to now there is only one study using contrast-enhanced endoscopic ultrasound with a 2nd generation contrast agent^[13]. Rickes *et al*^[27] have reported a similar finding.

In addition to their observations, we observed contrast enhancement of the 2nd generation contrast agent SonoVue® in both benign and malignant pancreatic lesions. To confirm our results we used endosonography-guided fine needle aspiration of the lesions. Endoscopic ultrasound-guided fine needle aspiration has become a highly valuable technique for acquisition of cytologic specimens in pancreatic diseases in the last 10 years. False-positive EUS-FNA cytologies do occur but seldom^[28] in large series of EUS-FNA for pancreatic masses. EUS-FNA provides a cytologic diagnosis of 80%-94% malignancies^[29-32].

In our study, the successful rate was 90% (successful fine needle aspiration of pancreatic masses in 86 of 96 patients), which is in accordance with the literature. The 22 G fine needle aspiration system allowed histological examination of the specimens in 8% of the cases. Up to now no patient with chronic pancreatitis developed pancreatic cancer during the 12-mo follow-up. The cytological results could be confirmed in all the 19 patients undergone surgery.

The method is limited by hindered passage through the pylorus and depth of penetration. As all the new methods are concerned, a certain training factor has to be taken into account since a steady and targeted transducer manipula-

tion is the prerequisite for optimal image documentation. Sometimes, severe lipomatosis of pancreas reduces image quality. It must be mentioned that the best results can be achieved using the present method. For equal results in modern sonographic systems, changes in the pre-settings of colour Doppler are required.

To date, ultrasonography is the most commonly used imaging modality in many countries. The advantages of endoscopic ultrasound (e.g. lack of radiation exposure, relatively low cost) are countered by its operator-dependency. Endoscopic ultrasound using contrast-enhancing agents may improve our understanding of the morphologic imaging methods through additional analyses of the functional criteria like perfusion with better characterization of tumours, lymph node staging and organ infiltration. The improved image (video) documentation enhances confidence in the method.

In conclusion, contrast-enhanced endoscopic ultrasound improves the differentiation between chronic pancreatitis and pancreatic carcinoma and seems to be a useful method in clinical practice.

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Effect of melatonin on the severity of L-arginine-induced experimental acute pancreatitis in rats

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Abstract

AIM: To determine the effect of melatonin pre- and post-treatment on the severity of L-arginine (L-Arg) -induced experimental pancreatitis in rats.

METHODS: Male Wistar rats (25) were divided into five groups. Those in group A received two injections of 3.2 g/kg body weight L-Arg i.p. at an interval of 1 h. In group MA, the rats were treated with 50 mg/kg body weight melatonin i.p. 30 min prior to L-Arg administration. In group AM, the rats received the same dose of melatonin 1 h after L-Arg was given. In group M, a single dose of melatonin was administered as described previously. In group C the control animals received physiological saline injections i.p. All rats were exsanguinated 24 h after the second L-Arg injection.

RESULTS: L-Arg administration caused severe necrotizing pancreatitis confirmed by the significant elevations in the serum amylase level, the pancreatic weight/body weight ratio (pw/bw), the pancreatic IL-6 content and the myeloperoxidase activity, relative to the control values. Elevation of the serum amylase level was significantly reduced in rats given melatonin following L-Arg compared to rats injected with L-Arg only. The activities of the pancreatic antioxidant enzymes (Cu/Zn-superoxide dismutase (Cu/Zn-SOD) and catalase (CAT)) were significantly increased 24 h after pancreatitis induction. Mela-

tonin given in advance of L-Arg significantly reduced the pancreatic CAT activity relative to that in the rats treated with L-Arg alone. In the liver, L-Arg significantly increased the lipid peroxidation level, and the glutathione peroxidase and Cu/Zn-SOD activities, whereas the Mn-SOD activity was reduced as compared to the control rats. Melatonin pre-treatment prevented these changes.

CONCLUSION: Melatonin is an antioxidant that is able to counteract some of the L-Arg-induced changes during acute pancreatitis, and may therefore be helpful in the supportive therapy of patients with acute necrotizing pancreatitis.

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Key words: Acute pancreatitis; Melatonin; Scavengers

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INTRODUCTION

Acute necrotizing pancreatitis is a disease with a high mortality and no efficient treatment is available for it at present. Oxygen- and nitrogen-derived free radicals (FRs) and lipid peroxidation play an important role in the development of local inflammation and systemic complications during acute pancreatitis^[1-6]. They damage the lipid membranes, structural and enzymatic proteins and DNA of the cells. The major target of FRs is polyunsaturated fatty acids of the lipid-rich membranes. Lipid peroxidation results in loss of the membrane fluidity and integrity, leading to cell death. Numerous antioxidants have recently been examined for their protective properties against oxidative damage in acute pancreatitis and have been shown to moderate the changes in several parameters of the disease^[7-10]. We presumed that a compound with strong antioxidant properties and also anti-inflammatory features might exert a beneficial effect on the outcome of severe experimental pancreatitis.

The pineal product, melatonin, is known to play a role

in the regulation of circadian rhythm and in the seasonal reproduction of certain species. Melatonin is also important as regards the physiology of the retina and the immune system^[11]. The antioxidant activity of melatonin has recently received significant attention. Although its level is low in the blood, a strong correlation has been observed between this level and the antioxidant capacity of serum both *in vitro* and *in vivo*^[12, 13]. Since melatonin is strongly lipophilic, its intracellular concentration may be several times higher than that in serum^[14]. Various body fluids likewise contain melatonin levels that are orders of magnitude higher than those measured in blood^[15].

Melatonin can detoxify OH, ONOO-, NO and peroxy radicals directly by electron donation, stimulate the activities of scavenger enzymes, including glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT), and protect these proteins from inactivation by the above-mentioned radicals^[11, 16-18]. The inhibition of nitrogen monoxide synthase (NOS) by melatonin leads to a reduction in the amount of NO generated^[19, 20]. Moreover, melatonin exerts an anti-inflammatory effect by inhibiting nuclear factor kappa B (NF- κ B)^[21], a transcription factor with a central role in the development of inflammatory diseases. By blocking the activation of NF- κ B, melatonin depresses the synthesis of inflammatory cytokines such as interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-alpha) and adhesion molecules.

The protective effects of melatonin have been documented in experimental models of numerous diseases where oxidative damage is a component. In the central nervous system, melatonin is protective in experimental models of stroke, Alzheimer, Parkinson and Huntington diseases, and fetal brain injury, and improves the outcome of hypoxia/reperfusion-induced heart, liver, retina and gut injuries^[22-27]. Melatonin additionally contributes to the improvement of inflammatory diseases, including endotoxic and circulatory shock^[23, 28].

In an earlier study, Qi and colleagues^[29] showed that in mild cerulein-induced edematous pancreatitis pharmacological doses of melatonin protect against the injury caused by FRs. Melatonin decreased the edema in the pancreas and stomach, and also the extent of lipid peroxidation in the pancreas. Jaworek *et al*^[30] demonstrated that even the circadian changes in physiological levels of melatonin reduce the severity of experimental pancreatitis^[30].

L-Arg-induced pancreatitis is an experimental model of severe necrotizing acute pancreatitis. Twenty-four h after intraperitoneal (i.p.) injection of L-Arg, inflammation of the tissue is confirmed by histology and characteristic changes of the laboratory parameters. The model is highly reproducible, noninvasive and produces dose-dependent acinar necrosis, and is therefore ideal for studying the pathogenesis of acute pancreatitis^[31-35].

The protective effect of melatonin in severe L-Arg-induced pancreatitis in rats has not been investigated to date. We hypothesized that administration of pharmacological doses of melatonin might improve the outcome of L-Arg-induced necrotizing pancreatitis in rats. To test this, we measured a variety of parameters related to pancreatic damage by L-Arg when melatonin was given before or after L-Arg.

Table 1 Agents used in the 5 groups of rats

| Group | Time: 0 h | Time: 0,5 h | Time: 1,5 h | Time: 2,5 h | Time: 25,5 h |
|-------|-----------------------|----------------------|----------------------|-----------------------|----------------|
| C | P.s. 1 mL | P.s. 1 mL | P.s. 1 mL | | exsanguination |
| A | P.s. 1 mL | Arginine 3.2 g/kg | Arginine 3.2 g/kg | | exsanguination |
| AM | Arginine 3,2 g/kg | Arginine 3.2 g/kg | | Melatonin 50 mg/kg | exsanguination |
| MA | Melatonin 50 mg/kg | Arginine 3.2 g/kg | Arginine 3.2 g/kg | | exsanguination |
| M | Melatonin 50 mg/kg | P.s. 1mL | P.s. 1 mL | | exsanguination |

MATERIALS AND METHODS

Experimental protocol

Male Wistar rats (weighing 200-250 g) were kept at constant room temperature (25°C) in a 12h light-dark cycle with free access to water and standard laboratory chow (Biofarm, Zagyvaszárszó, Hungary). The study was approved by the Ethical Committee on Animal Experiments at the University of Szeged.

After one week of acclimatization, the rats were divided into five groups ($n=5$ per group). In group A, pancreatitis was induced with 3.2 g/kg body weight L-Arg (Sigma-Aldrich, Budapest, Hungary) i.p. twice at an interval of 1 h. Rats in group MA were treated with a single dose of 50 mg/kg body weight melatonin (Helsinn, Biasca, Switzerland) i.p. 30 min prior to L-Arg administration. Rats in group AM received the same dose of melatonin 1 h after the second injection of L-Arg. In group M, a single dose of melatonin was administered. Rats in group C served as control animals and received i.p. injections of physiological saline. Twenty-four h after the last L-Arg injection, the rats were anesthetized with 44 mg/kg pentobarbital (Sanofi Phylaxia, Budapest, Hungary) and exsanguinated through the abdominal aorta. The pancreas, liver and lungs were quickly removed and frozen at -70°C until use (Table 1).

Assays

The pancreatic weight/body weight ratio was evaluated as an estimate of the degree of pancreatic edema. For serum amylase activity, blood samples were centrifuged for 20 min at 2500 r/min. Serum amylase activities were determined by a colorimetric kinetic method (Dialab, Vienna, Austria). Concentration of the lipid peroxidation marker malonyl dialdehyde (MDA) was measured after the reaction with thiobarbituric acid as previously described^[36]. Total SOD activity was determined on the basis of the inhibition of epinephrine-adenochrome autoxidation^[37]. Mn-SOD activity was measured via the extent of autoxidation in the presence of 5×10^{-3} mol/L KCN^[38]. Cu/Zn-SOD activity was obtained by deducting the Mn-SOD from the total SOD activity. CAT activity was determined spectrophotometrically at 240 nm by the method of Beers and Sizer^[39] and expressed in Bergmeyer units (BU) (1 BU - decomposition of 1 g H₂O₂/min at 25°C). GPx activity was determined by the "chemical" method using cumene

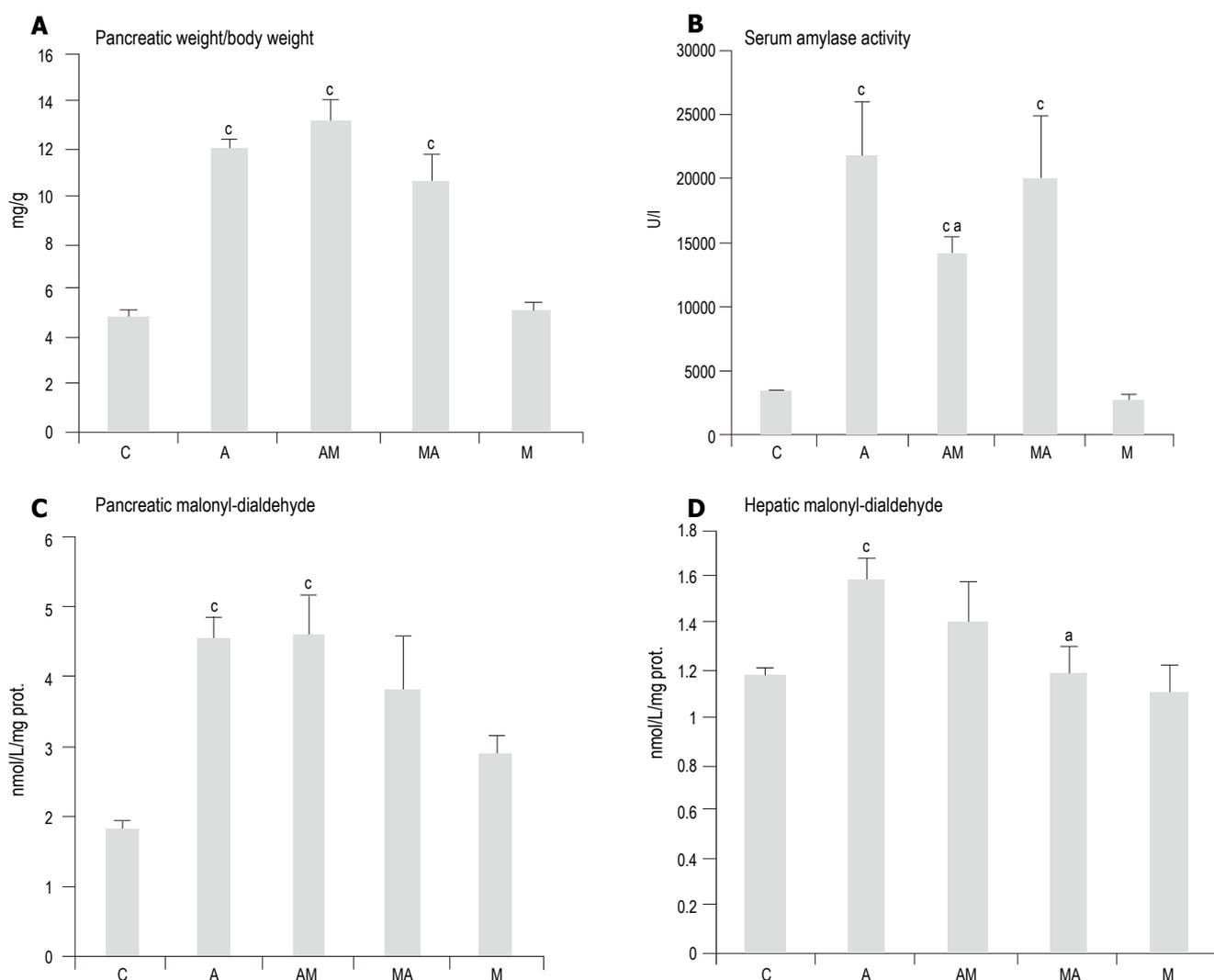


Figure 1 Effects of melatonin (50 mg/kg) treatment on the pancreatic weight/body weight ratio (p.w./b.w.) (A), serum amylase activity (B), amount of malonyl-dialdehyde in the pancreas (C) and liver (D) in L-arginine-induced (2 x 3.2 g/kg) acute pancreatitis. Means \pm SE of results on 5 animals in each group are shown. * $P < 0.05$ vs group A; † $P < 0.05$ vs group C.

hydroperoxide and reduced glutathione as substrates of GPx^[40]. Total glutathione (GSH) was measured spectrophotometrically with Ellman's reagent^[41]. The level of leukocyte infiltration into the tissue was quantified by measurement of pancreatic myeloperoxidase (MPO) activities by the method of Kuebler *et al.*^[42].

Preparation of cytosolic fraction

The cytosolic and nuclear fractions were separated by the method of Dignam *et al.*^[43]. In brief, 250-300 mg of pancreatic tissue was lysed on ice in a hypotonic buffer in a Dounce homogenizer. The buffer contained 10 mmol/L HEPES (pH 7.9), 1.5 mmol/L MgCl₂ 10 mmol/L KCl and was supplemented before use with 1 mmol/L phenylmethylsulfonyl fluoride, 4 mmol/L benzamidine, 100 IU/mL aprotinin and 1 mmol/L dithiothreitol (DTT). After incubation for 25 min on ice, Nonidet P-40 was added to a final concentration of 0.3-0.4% (v/v). The samples were vortexed and incubated on ice for 2 min. The homogenates were centrifuged at 13000 r/min for 50 s and the supernatants (cytosolic fraction) were collected for determination of protein and IL-6 concentrations.

Pancreatic cytokine concentration

Pancreatic IL-6 concentration in the cytosolic fraction was measured with an ELISA kit (Bender Medsystem, Vienna, Austria) according to the manufacturer's instructions and corrected for the protein content of the tissue. Protein concentration in the tissues was determined by the method of Lowry *et al.*^[44].

Histological examination

A portion of the pancreas was fixed in 8% neutral formaldehyde solution and embedded in paraffin. Tissue slices were stained with hematoxylin and eosin and examined under light microscope. Slices were coded and examined blind by a pathologist for the grading of histological alterations.

Statistical analysis

One-way ANOVA and LSD *post hoc* analysis were performed to test for significant differences among the five experimental groups. Results were expressed as mean \pm SE. $P < 0.05$ was considered statistically significant.

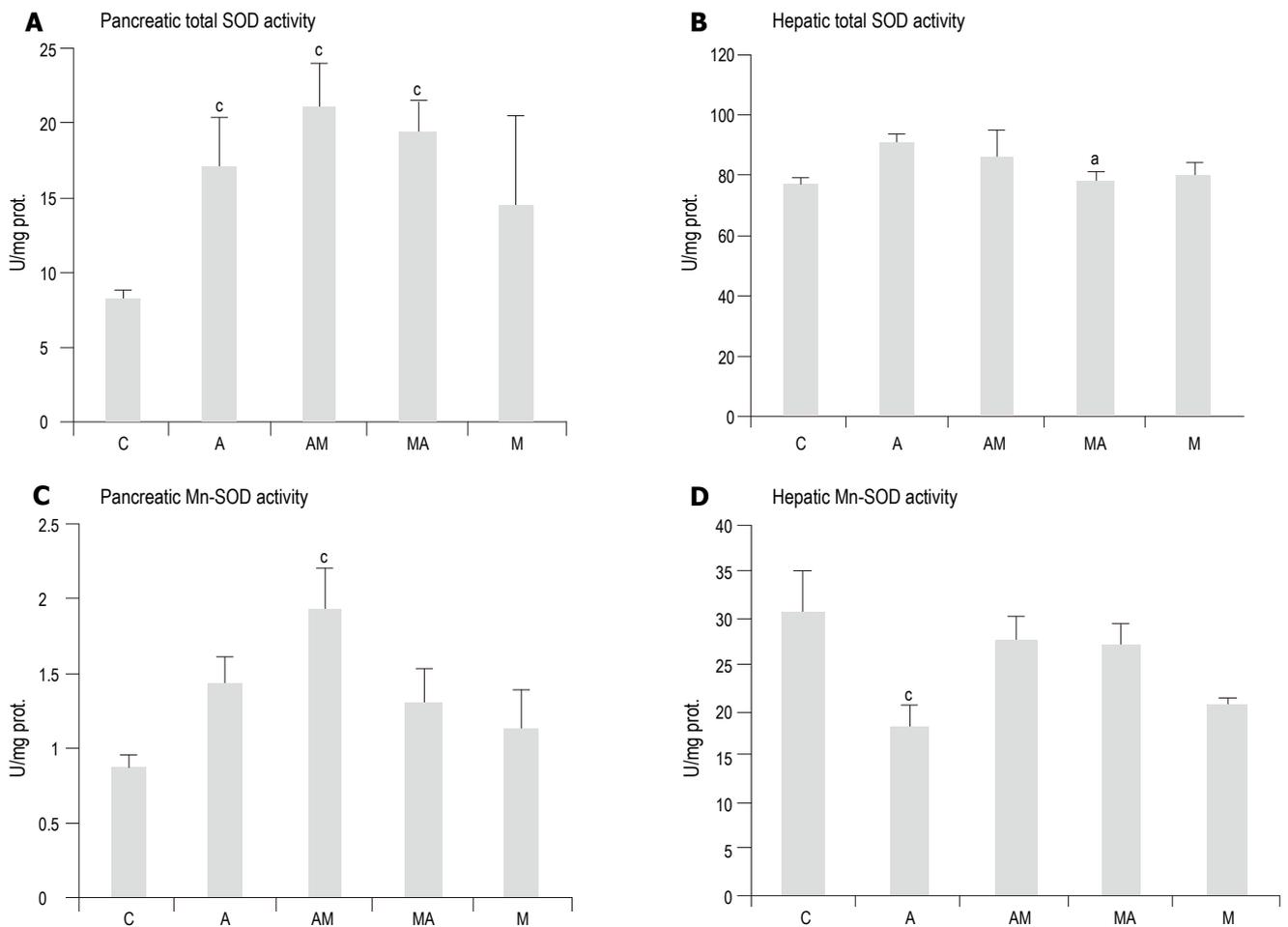


Figure 2 Effects of melatonin (50 mg/kg) treatment on the total SOD and Mn-SOD activities of the pancreas (A, C) and liver (B, D) in L-arginine-induced (2 x 3.2 g/kg) acute pancreatitis. Means \pm SE of results on 5 animals in each group are shown. ^a $P < 0.05$ vs group A; ^c $P < 0.05$ vs group C.

RESULTS

Melatonin treatment alone caused no significant alterations in the measured parameters as compared to those in the control rats.

The pancreatic weight/body weight ratio was significantly higher in the L-Arg-treated rats than in the control group. Melatonin given before or after L-Arg, did not influence the degree of edema (Figure 1A).

The serum amylase activity in all L-Arg-injected rats was significantly elevated relative to the control. Melatonin posttreatment significantly reduced the amylase activity as compared to the treatment only with L-Arg (Figure 1B).

The concentration of lipid peroxidation product MDA in the pancreas was increased in the rats treated with L-Arg or with L-Arg followed by melatonin. When melatonin was given before L-Arg, the MDA level was not significantly changed compared to that in the control group (Figure 1C). The amount of MDA in the liver of animals given L-Arg was significantly increased compared to that in the control group. Melatonin pretreatment significantly reduced the concentration of the lipid peroxidation product (Figure 1D).

The pancreatic total SOD activity was significantly increased as a result of L-Arg administration and the effect was not modified by pre- or post-treatment with melatonin

(Figure 2A). The hepatic total SOD activities in the L-Arg-treated groups were not significantly different from those in the control rats, but the L-Arg-treated rats having previously received melatonin exhibited a significantly lower SOD activity than those injected only with L-Arg (Figure 2B).

Relative to the level in the control rats, the pancreatic Mn-SOD activity was significantly elevated in animals given L-Arg followed by melatonin (Figure 2C). The Mn-SOD activity in the liver was significantly decreased as a consequence of L-Arg injections. Melatonin given before or after L-Arg prevented the reduction of Mn-SOD activity (Figure 2D).

As compared to the levels in the control rats, the pancreatic Cu/Zn-SOD activities were significantly elevated in all L-Arg-treated groups (Figure 3A). Relative to the control levels, the hepatic Cu/Zn-SOD activity was significantly increased in rats that received only L-Arg, this change was prevented by melatonin given before or after the L-Arg injections (Figure 3B).

The pancreatic CAT activity was significantly increased in the rats given only L-Arg injections and also in those given L-Arg followed by melatonin, relative to that in the control rats. However, in the rats pretreated with melatonin the changes in CAT activity were prevented (Figure 3C). As compared to the level in the control rats, the hepatic

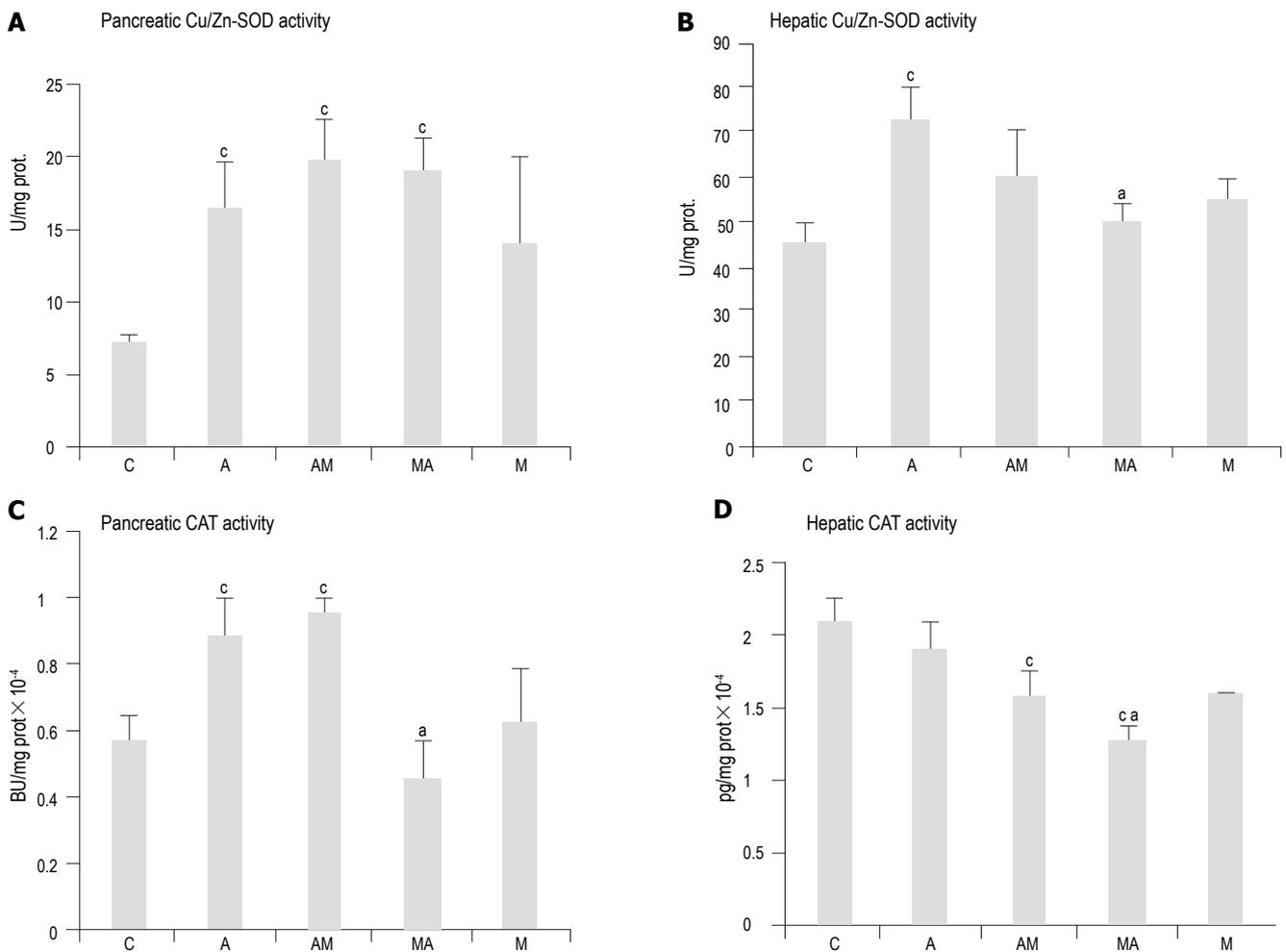


Figure 3 Effects of melatonin (50 mg/kg) treatment on the Cu/Zn-SOD and CAT activities of the pancreas (A, C) and liver (B, D) in L-arginine-induced (2 x 3.2 g/kg) acute pancreatitis. Means \pm SE of results on 5 animals in each group are shown. ^a $P < 0.05$ vs group A; ^c $P < 0.05$ vs group C.

CAT activities were significantly reduced in the rats treated with melatonin before or after L-Arg, whereas in the rats given only L-Arg the CAT activity was unchanged (Figure 3D).

The pancreatic GSH level was not significantly influenced by any of the treatments (Figure 4A). GPx activity was not detectable in the pancreas of the rats in this study. In comparison with the level in the control rats, hepatic GPx activities were significantly elevated in the rats treated only with L-Arg and in the rats given melatonin following L-Arg, but not in the rats pretreated with melatonin (Figure 4B).

The pancreatic MPO activity was significantly increased in the L-Arg-treated rats compared to that in the control rats. This response was reduced by melatonin given before or after L-Arg injections (Figure 4C).

Relative to the level in the control rats, the pancreatic IL-6 concentration was significantly elevated in the L-Arg-injected animals (Figure 4D).

Histological investigation confirmed the development of severe necrotizing pancreatitis in all rats given L-Arg, with no discernible differences between the groups.

DISCUSSION

This study demonstrated the antioxidant effect of

melatonin during L-Arg-induced severe necrotizing pancreatitis. The dose of 50 mg/kg melatonin was chosen according to literary data and a pilot-study investigating the effect of different doses of melatonin on the pancreatic weight/body weight ratio and the serum amylase activity [22,28-29]. In contrast to Qi and colleagues who used repeated injections of melatonin, we could detect the beneficial effect of the drug after administration of a single dose in the same order of magnitude [29]. Melatonin beneficially influenced the serum amylase activity, the free radical scavenger enzyme activity in the liver and pancreas as well as the lipid peroxidation level in the liver.

L-Arg-induced pancreatitis is a slowly-developing experimental model in which characteristic laboratory changes are observed 24 h after induction of the disease. By this time, administration of high doses of L-Arg can cause severe necrotizing pancreatitis, as observed in the current study and confirmed by the significant elevation of the serum amylase activity, edema of the pancreas and the increased level of lipid peroxidation marker MDA. The significantly higher MPO activity and the increased amount of pro-inflammatory cytokine IL-6 in the pancreas document the initiation of an inflammatory process in the pancreas.

Elevation of the serum amylase activity is one of the characteristic parameters of acute pancreatitis. The activ-

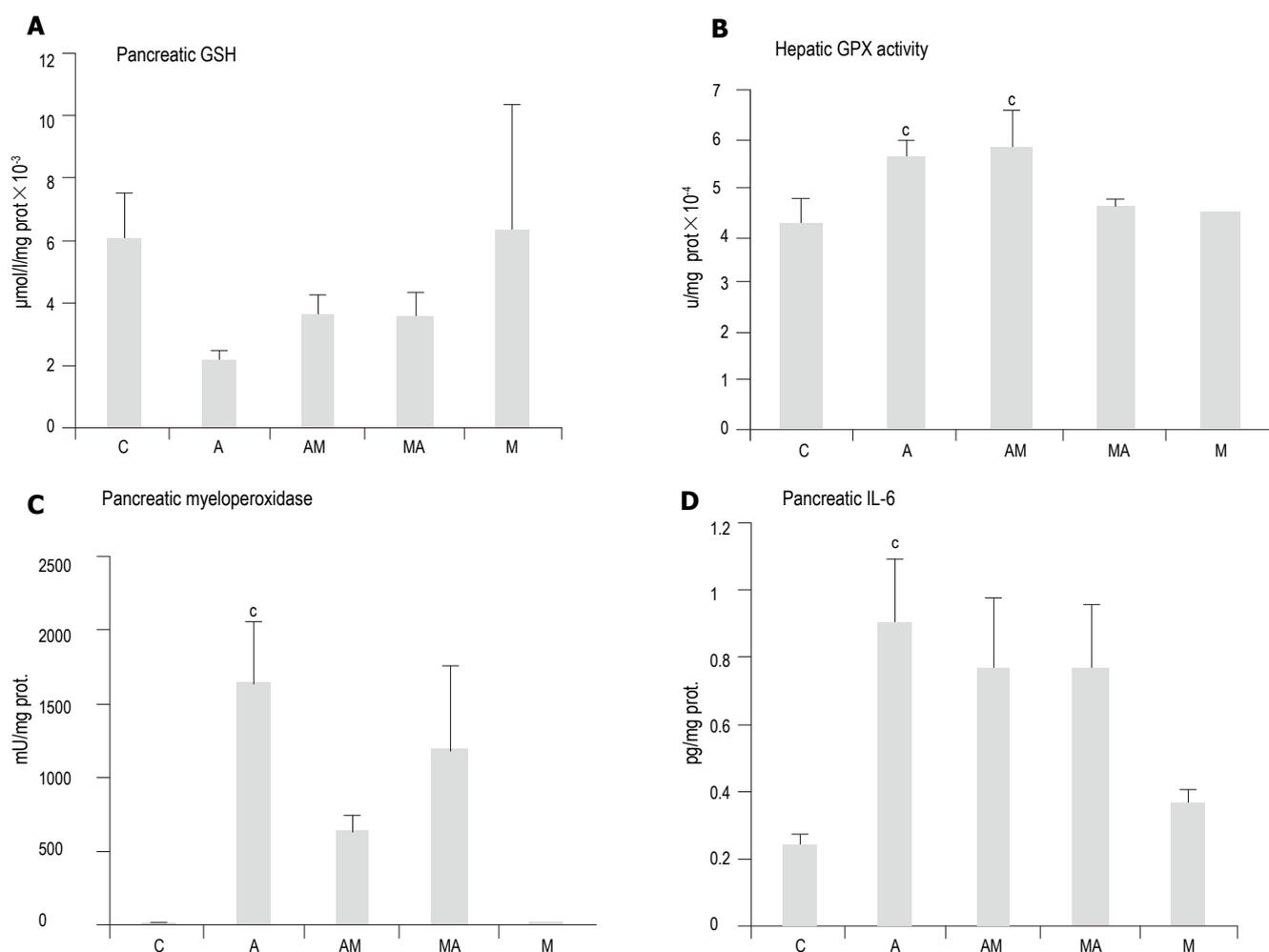


Figure 4 Effects of melatonin (50 mg/kg) treatment on the pancreatic GSH content (A), liver GPx activity (B), pancreatic myeloperoxidase activity (C) and pancreatic IL-6 content (D) in L-arginine-induced (2 × 3,2 g/kg) acute pancreatitis. Means ± SE of results on 5 animals in each group are shown. ^a*P*<0.05 vs group A; ^c*P*<0.05 vs group C.

ity of this enzyme begins to rise 12 h after administration of L-Arg and peaks at around 24 h. At this point, the level of serum amylase activity correlates with the severity of acinar destruction. In our experiment administration of melatonin following the induction of pancreatitis can significantly reduce the activity of this enzyme in serum.

Infiltrating leukocytes and mediators released by these cells are known to play a pivotal role in the amplification of the inflammatory process. One of the inflammatory cytokines that is important in the development of L-Arg-induced pancreatitis is IL-6. In the present study, the level of IL-6 and the MPO activity proved to be significantly elevated in the L-Arg-treated rats. However, the previously reported anti-inflammatory effect of melatonin could not be clearly demonstrated in severe necrotizing pancreatitis [45].

Lipid peroxidation in the pancreas and distant organs is a process mediated by free radicals. Detection of these radicals is difficult because of their high reactivity and short half-life. Accordingly, we measured the activities of free radical scavengers and the degree of lipid peroxidation in order to assess the extent of free radical damage during this inflammatory process. In our experimental setting we did not focus on the detection of early events of acute pancreatitis, such as decreased scavenger enzyme activities

and enzyme-activating effects of melatonin, which took place during the first 6-12 h of the disease. We rather wanted to demonstrate the long-lasting effect of a single dose of melatonin on the fully developed illness.

The current study revealed that melatonin pretreatment significantly attenuated the lipid peroxidation in the liver of the rats. Changes in Cu/Zn-SOD, Mn-SOD and GPx activities in the liver were reduced by melatonin, whereas in the pancreas the beneficial effect was less pronounced and manifested only in the changes of CAT activity. In the pancreas, H₂O₂ generated by SOD can serve as a substrate of GPx or CAT. This explains the CAT activity elevation and the lack of GPx activity in the pancreas. The differences observed between the examined organs can be explained by the fact that the basal activities of scavenger enzymes are 10-fold higher in the liver than in the pancreas. The antioxidant effect of melatonin is therefore easier to demonstrate in the liver of animals. These differences in scavenger activities explain why melatonin pretreatment can reduce the lipid peroxidation in the liver, but not in the pancreas. The low scavenger activities are probably one reason for the high mortality associated with acute pancreatitis.

In conclusion, melatonin is able to counteract some of the L-Arg-induced changes in laboratory parameters of

acute pancreatitis. Even a single injection of melatonin can beneficially influence serum amylase activity and lipid peroxidation level in the liver, but can not prevent histological damage to the pancreas. Since melatonin has a very short half-life, repeated injections or continuous infusion may be necessary to develop its full effect. Multiple organ failure is the main reason for pancreatitis-associated mortality. As melatonin reduces hepatic damage caused by L-Arg, it is possible that continuous infusion of the substance may be beneficial in preventing multiple organ damage as a complication of acute necrotizing pancreatitis.

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Antioxidative effect of melatonin, ascorbic acid and *N*-acetylcysteine on caerulein-induced pancreatitis and associated liver injury in rats

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Abstract

AIM: To investigate the role of oxidative injury in pancreatitis-induced hepatic damage and the effect of antioxidant agents such as melatonin, ascorbic acid and *N*-acetyl cysteine on caerulein-induced pancreatitis and associated liver injury in rats.

METHODS: Thirty-eight female Wistar rats were used. Acute pancreatitis (AP) was induced by two i.p. injections of caerulein at 2-h intervals (at a total dose of 100 µg/kg b.wt). The other two groups received additional melatonin (20 mg/kg b.wt) or an antioxidant mixture containing L(+)-ascorbic acid (14.3 mg/kg b.wt.) and *N*-acetyl cysteine (181 mg/kg b.wt.) i.p. shortly before each injection of caerulein. The rats were sacrificed by decapitation 12 h after the last injection of caerulein. Pancreatic and hepatic oxidative stress markers were evaluated by changes in the amount of lipid peroxides measured as malondialdehyde (MDA) and changes in tissue antioxidant enzyme levels, catalase (CAT) and glutathione peroxidase (GPx). Histopathological examination was performed using scoring systems.

RESULTS: The degree of hepatic cell degeneration, intracellular vacuolization, vascular congestion, sinusoidal dilatation and inflammatory infiltration showed a significant difference between caerulein and caerulein+melatonin ($P=0.001$), and caerulein and caerulein+L(+)-ascorbic acid+*N*-acetyl cysteine groups ($P=0.002$). The degree of aciner cell degeneration, pancreatic edema, intracellular vacuolization and inflammatory infiltration showed a significant difference between caerulein and

caerulein+melatonin ($P=0.004$), and caerulein and caerulein+L(+)-ascorbic acid+*N*-acetyl cysteine groups ($P=0.002$). Caerulein-induced pancreatic and liver damage was accompanied with a significant increase in tissue MDA levels ($P=0.01$, $P=0.003$, respectively) whereas a significant decrease in CAT ($P=0.002$, $P=0.003$, respectively) and GPx activities ($P=0.002$, $P=0.03$, respectively). Melatonin and L(+)-ascorbic acid+*N*-acetyl cysteine administration significantly decreased MDA levels in pancreas ($P=0.03$, $P=0.002$, respectively) and liver ($P=0.007$, $P=0.01$, respectively). Administration of these agents increased pancreatic and hepatic CAT and GPx activities. Melatonin significantly increased pancreatic and hepatic CAT ($P=0.002$, $P=0.001$, respectively) and GPx activities ($P=0.002$, $P=0.001$). Additionally, L(+)-ascorbic acid+*N*-acetyl cysteine significantly increased pancreatic GPx ($P=0.002$) and hepatic CAT and GPx activities ($P=0.001$, $P=0.007$, respectively).

CONCLUSION: Oxidative injury plays an important role not only in the pathogenesis of AP but also in pancreatitis-induced hepatic damage. Antioxidant agents such as melatonin and ascorbic acid+*N*-acetyl cysteine, are capable of limiting pancreatic and hepatic damage produced during AP via restoring tissue antioxidant enzyme activities.

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Key words: Caerulein; Liver; Melatonin; Oxidative stress; Pancreatitis

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INTRODUCTION

Acute pancreatitis (AP) is an inflammatory disease characterized by tissue edema, acinar necrosis, hemorrhage and fat necrosis as well as inflammation and perivascular infiltration in the pancreas^[1,2]. In experimental pancreatitis induced by supramaximal doses of the cholecystokinin (CCK) analogue, caerulein, the secretory block is subse-

quently followed by lysosomal degradation of intercellular organelles within autophagic vacuoles in acinar cells and a marked interstitial edema^[3-6]. AP is usually considered as an autodigestive disease, in which in addition to premature intracellular protease activation^[7], other mechanisms such as oxidative stress have also been shown to be involved in the development of AP^[6,8-11].

AP is a multi-system disease with alterations not only in the pancreas but also in liver, lungs and kidneys, which may lead to distant organ dysfunction and death^[12,13]. The liver is a critical organ for metabolic homeostasis and toxic substance clearance and plays an important role in the systemic response to critical illness^[14]. Recently, a number of studies have suggested that pancreatitis-associated ascitic fluid (PAAF) plays a critical role in inducing hepatocyte injury by inducing hepatocyte apoptosis^[14-16]. PAAF induces liver injury by direct hepatocyte injury and death independent from locally produced Kupffer-cell-derived cytokines and PAAF-induced liver injury is mediated by heat-stable factors other than pancreatic enzymes^[16]. Though the role of oxidative stress in AP has been studied in several animal models^[2,6,11,17] and in humans^[18], few data are available regarding AP-induced hepatic oxidative stress^[19].

Previous reports have indicated that antioxidant agents such as melatonin, retinol, ascorbic acid and *N*-acetyl cysteine have beneficial effects in the treatment of caerulein-induced AP^[18,20-22]. This study was undertaken to examine the role of oxidative stress in the pathogenesis of AP and AP-induced hepatic damage as well as the protective effect of antioxidant agents against caerulein-induced pancreatic and liver injury.

MATERIALS AND METHODS

Animals and experimental protocol

Thirty-eight female Wistar rats weighing 280-350 g were used. Animals were fed with standard rat chow and tap water *ad libitum*. They were maintained in a 12 h light/12 h dark cycle at 21°C.

AP was induced by two i.p. injections of caerulein (Sigma-Aldrich Co., Taufkirchen, Germany) at a total dose of 100 µg/kg b.wt. at 2-h intervals, each injection containing 50 % of the dose. A control group received four i.p. injections of 0.9% saline at 2-h intervals. To evaluate the effect of antioxidant agents, the rats were divided into two additional groups. One group was treated with caerulein (100 µg/kg b.wt) and melatonin (Sigma, St Louis, MO, USA) (20 mg/kg b.wt) and the other group was treated with the same dose of caerulein and an antioxidant mixture containing L(+)-ascorbic acid (Mallinckrodt Baker B.V., Deventer, Holland) (14.3 mg/kg b.wt.) and *N*-acetyl cysteine (Sigma, St. Louis MO, USA) (181 mg/kg b.wt.). Melatonin was dissolved in absolute ethanol and further diluted in saline, with 1 % final concentration of ethanol. Therapeutic agents were administered i.p. shortly before each injection of caerulein. The rats were sacrificed by decapitation 12 h after the last injection of caerulein.

For light microscopy, pieces from central part of the pancreas and right lobe of the liver were rapidly removed and divided into two pieces. The first part of

tissue samples was placed in 10% buffered formalin and prepared for routine paraffin embedding. The other part of tissue samples was stored at -80°C for determination of MDA, GPx and CAT.

Animal experiments were performed in accordance with the guidelines for animal research from the National Institute of Health and approved by the Committee of Animal Research at Inonu University, Malatya, Turkey.

Histological examination

Five-µm thick sections of tissues were cut, mounted on slides, stained with hematoxylin-eosin (H-E) and examined under a Lyca DFC280 light microscope by Leica Q Win and Image Analysis System (Leica Micros Imaging Solutions Ltd.; Cambridge, U.K). Assessment of tissue alterations in 20 different fields for each section was conducted by an experienced histologist who was unaware of the treatment. Pancreatic damage was scored by grading acinar cell degeneration, interstitial inflammation, edema and hemorrhage with a maximum score of 12. Schmidt's standards^[1] were modified as follows. Grading for edema was scaled as 0: absent or rare; 1: edema in the interlobular space; 2: edema in the intralobular space; 3: isolated-island shape of pancreatic acinus. Inflammation was scored as 0: absent; 1: mild; 2: moderate; 3: severe. Acinar cell degeneration was scaled as 0: absent; 1: focal (<5 %); 2: and/or sublobular (<20 %); 3: and/or lobular (>20 %). Parenchyma hemorrhage was scored as 0: absent; 1: mild; 2: moderate; 3: severe. Hepatic damage was scored (0 to 3) by grading hepatocyte necrosis, intracellular vacuolization, vascular congestion and sinusoidal dilatation. The maximum score for inflammatory infiltration was 12.

Biochemical determination

After decapitation, the trunk blood was collected and centrifuged for measurements of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactic dehydrogenase (LDH), total and direct bilirubin. Amylase. Lipase levels were determined spectrophotometrically using an automated analyzer (Olympus AU 600, Diamond Diagnostic, Holliston, USA). All chemicals were obtained from Sigma (Sigma, St Louis MO, USA).

Preparation of tissue homogenates

After being cut into small pieces on ice, tissues were homogenized in 1/5 (w/v) phosphate-buffered saline. Homogenates were divided into two portions. One part was directly used for immediate MDA measurement. The other part was sonicated four times for 30 s at 20 s intervals using a VWR Bronson scientific sonicator (VWR Int. Ltd. Merch House Pool, UK). Then, homogenates were centrifuged at 20000 g for 15 min in Beckman L-8-70M ultracentrifuge (Rotor SW-28; Beckman L8-70M Ultracentrifuge, München, Germany). Supernatants were separated and kept at -40°C for enzyme activity measurement. Care was taken to keep the temperature at -4°C throughout the preparation of homogenates and supernatants.

Determination of protein concentration

Protein determination in supernatants was done according to Lowry and Rosebrough^[23] using BSA as standard. A

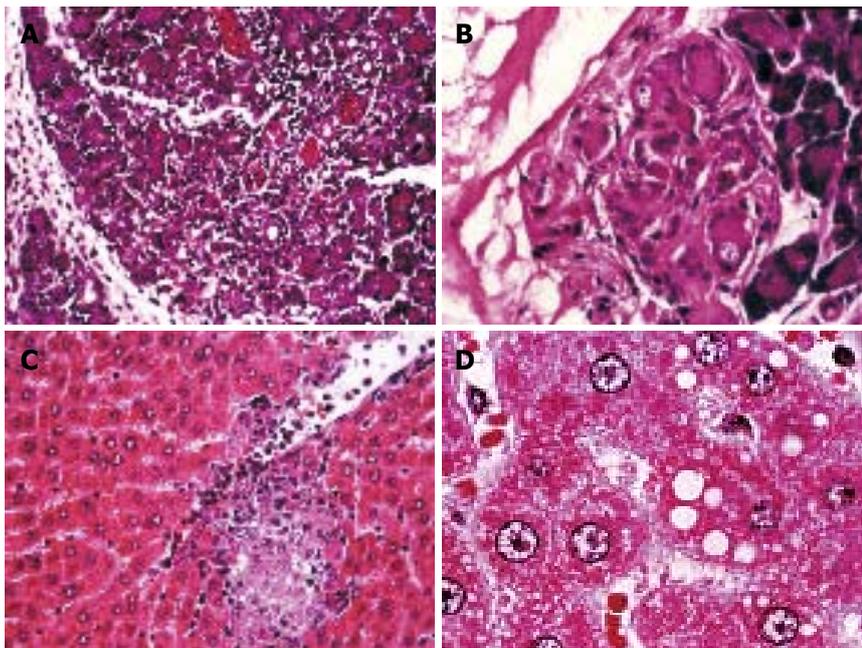


Figure 1 Histopathological changes in pancreatic and hepatic specimens

A: Obvious acinar cell degeneration, edema and inflammation in caerulein group.

B: Acinar cell degeneration and fat necrosis in caerulein group.

C: Hepatocyte necrosis, vascular congestion, sinusoidal dilatation and inflammatory infiltration in caerulein group.

D: Prominent intracellular vacuolization in hepatic specimens from caerulein group.

Table 1 Scores for pancreatic and hepatic damage in different groups (mean \pm SE)

| Group | Scores for pancreatic damage | Scores for hepatic damage |
|---------------------------------|------------------------------|------------------------------|
| Control | 0.00 \pm 0.00 | 0.00 \pm 0.00 |
| Caerulein | 6.16 \pm 0.67 ^a | 6.28 \pm 0.56 ^a |
| Caerulein + melatonin | 2.14 \pm 0.70 ^c | 1.14 \pm 0.26 ^c |
| Caerulein + antioxidant mixture | 1.85 \pm 0.45 ^c | 1.42 \pm 0.36 ^c |

^a $P < 0.005$ vs control; ^c $P < 0.005$ vs caerulein.

Shimadzu 1601 UV/VIS spectrophotometer (Shimadzu, Kiyoto, Japan) with a connected PC and a Grand LTD 6G thermo stability unit adjusted to $37 \pm 0.1^\circ\text{C}$ was employed for all spectrophotometric assays.

Assay of CAT activity

CAT activity was measured in supernatants by the method of Luck^[24]. The decomposition of the substrate H_2O_2 was monitored spectrophotometrically at 240 nm. Specific activity was defined as micromole substrate decomposed per minute per milligram of protein (i.e. U/mg protein). CAT levels were expressed as a micromole per milligram of protein (U/mg/protein).

Assay of GPx activity

GPx activity was measured according to Lawrence and Burk^[25]. In brief, 1.0 mL of 50 nmol/L PBS solution (pH 7.4) including 5 mmol/L EDTA, 2 $\mu\text{mol/L}$ NADPH, 20 $\mu\text{mol/L}$ GSH, 10 $\mu\text{mol/L}$ NaN_3 and 23 mU of glutathione reductase was incubated at 37°C for 5 min. Then 20 μL of 0.25 mmol/L H_2O_2 solution and 10 μL of supernatant were added to the assay mixture. The change in absorbance at 340 nm was monitored for 1 min. A blank with all ingredients except for supernatants was also monitored. Specific activity was calculated as micromole NADPH consumed

per minute per milligram of protein (i.e. U/mg protein) using an appropriate molar absorptivity coefficient ($6220 \text{ M}^{-1} \text{ cm}^{-1}$). GPx levels were expressed as a micromole per milligram of protein (U/mg protein).

Measurement of tissue MDA levels

The level of MDA in tissue homogenate was determined using the method of Uchiyama and Mihara^[26]. Half a milliliter of homogenate was mixed with 3 mL H_3PO_4 solution (1 % v/v) followed by addition of 1 mL thiobarbituric acid solution (0.67 % w/v). Then the mixture was heated in water bath for 45 min. The colored complex was extracted into n-butanol and absorption at 532 nm was measured using tetramethoxypropane as standard. MDA levels were expressed as a nanomol per milligram of protein (nm/mg protein).

Statistical analysis

Statistical analysis was carried out using the SPSS 10.0 statistical program (SPSS Inc., Chicago, IL, USA). All data were expressed as mean \pm SE. For the analysis of histological scores and enzyme levels Mann Whitney-U test and *t* test were used. $P < 0.05$ was considered statistically significant.

RESULTS

All animals survived until the end of the experiment. Animals from control group presented no histological alterations. The pancreatic specimens from the caerulein treated group showed some histopathological changes such as acinar cell degeneration, edema and inflammation (Figure 1A, Figure 1B). The liver specimens from this group also showed histopathological alterations such as hepatocyte necrosis and intracellular vacuolization vascular congestion, sinusoidal dilatation and inflammatory infiltration (Figure 1C, Figure 1D). Histopathological scores of the groups are summarized in Table 1. In the groups treated

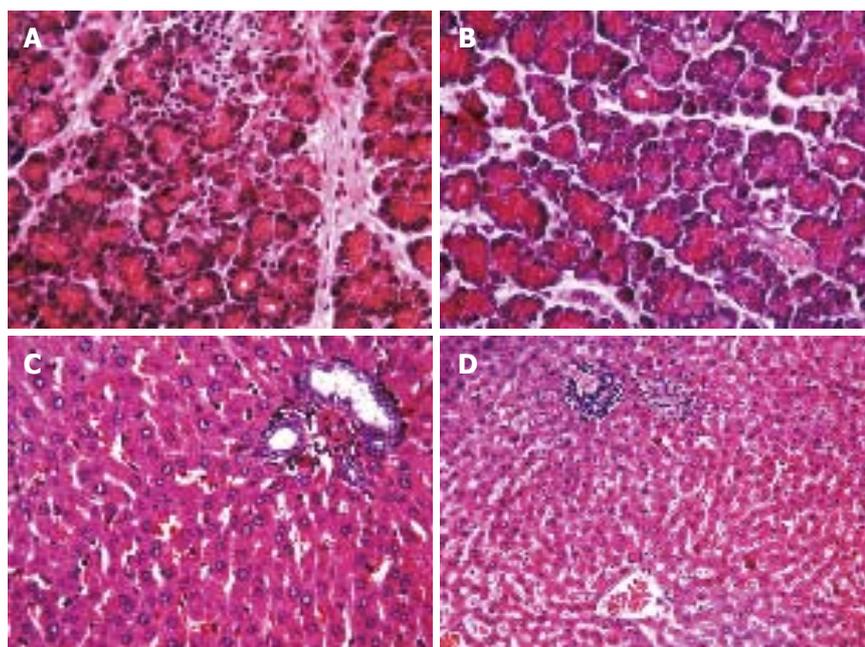


Figure 2 Histopathological evidence of pancreatic and hepatic damage

A: Normal histological appearance except for minimal infiltration in caerulein + melatonin group.

B: Normal histological appearance except for minimal edema in caerulein + L(+)-ascorbic acid + N-acetyl cysteine group.

C: Normal histological appearance in caerulein + melatonin group.

D: Two small areas of necrosis and cell infiltration in caerulein + L(+)-ascorbic acid + N-acetyl cysteine group.

Table 2 MDA, CAT and after treatment GPx levels in pancreatic tissues of different groups after treatment (mean \pm SE)

| Groups | MDA (nmol/mg pr) | CAT (U/mg pr) | GPx (U/mg pr) |
|---------------------------------|------------------------------|-------------------------------|------------------------------|
| Control | 1.74 \pm 0.23 | 10.29 \pm 0.68 | 0.59 \pm 0.05 |
| Caerulein | 2.63 \pm 0.22 ^a | 2.78 \pm 0.28 ^b | 0.10 \pm 0.00 ^b |
| Caerulein + melatonin | 1.90 \pm 0.17 ^c | 15.63 \pm 0.67 ^e | 1.72 \pm 0.29 ^e |
| Caerulein + antioxidant mixture | 1.30 \pm 0.15 ^e | 7.02 \pm 1.84 | 1.15 \pm 0.18 |

^a*P* < 0.05 vs control; ^c*P* < 0.05 vs caerulein; ^b*P* < 0.005 vs caerulein; ^e*P* < 0.005 vs control.

with caerulein + melatonin and caerulein + L(+)-ascorbic acid + N-acetyl cysteine, histopathological evidence of pancreatic and hepatic damage was markedly reduced (Figure 2). The degree of hepatic cell degeneration, intracellular vacuolization, vascular congestion, sinusoidal dilatation and inflammatory infiltration showed a significant difference between groups treated with caerulein and caerulein + melatonin (*P* = 0.001), caerulein and caerulein + L(+)-ascorbic acid + N-acetyl cysteine (*P* = 0.002). The degree of acinar cell degeneration, pancreatic edema, intracellular vacuolization and inflammatory infiltration showed a significant difference between groups treated with caerulein and caerulein + melatonin (*P* = 0.004), caerulein and caerulein + L(+)-ascorbic acid + N-acetyl cysteine (*P* = 0.002). Caerulein-induced pancreatitis and liver damage were accompanied with a significant increase in tissue MDA levels (*P* = 0.01, *P* = 0.003, respectively) whereas a significant decrease in CAT (*P* = 0.002, *P* = 0.003, respectively) and GPx activities (*P* = 0.002, *P* = 0.03, respectively). MDA, CAT and GPx levels measured in pancreatic and hepatic tissues of all groups are summarized in Tables 2 and 3 respectively. Administration of melatonin and L(+)-ascorbic acid + N-acetyl cysteine significantly decreased

Table 3 MDA, CAT and GPx levels in liver tissues of different groups after treatment (mean \pm SE)

| Group | MDA (nmol/mg pr) | CAT (U/mg pr) | GPx (U/mg pr) |
|---------------------------------|--------------------------------|------------------------------|------------------------------|
| Control | 7.00 \pm 2.29 | 3.20 \pm 0.83 | 0.72 \pm 0.25 |
| Caerulein | 20.89 \pm 10.13 ^a | 1.09 \pm 0.35 ^a | 0.33 \pm 0.09 ^b |
| | 9.66 \pm 3.65 ^c | 3.69 \pm 1.35 ^e | 0.50 \pm 0.12 ^c |
| Caerulein + antioxidant mixture | 10.49 \pm 3.03 ^c | 3.12 \pm 1.02 ^e | 0.61 \pm 0.26 ^c |

^a*P* < 0.005 vs control; ^c*P* < 0.05 vs caerulein; ^b*P* < 0.005 vs caerulein, ^e*P* < 0.05 vs control.

MDA levels in pancreas (*P* = 0.03, *P* = 0.002, respectively) and liver (*P* = 0.007, *P* = 0.01, respectively) while increased pancreatic and hepatic CAT and GPx activities. Melatonin significantly increased pancreatic and hepatic CAT (*P* = 0.002, *P* = 0.001, respectively) and GPx activities (*P* = 0.002, *P* = 0.001). Additionally, L(+)-ascorbic acid + N-acetyl cysteine significantly increased pancreatic GPx (*P* = 0.002) and hepatic CAT and GPx activities (*P* = 0.001, *P* = 0.007, respectively).

Caerulein administration resulted in a significant increase in serum AST (*P* < 0.02), ALT (*P* = 0.009), LDH (*P* = 0.002), total bilirubin (*P* = 0.001), direct bilirubin (*P* = 0.001), amylase (*P* = 0.02) and lipase (*P* = 0.002) levels (Tables 4 and 5). Both melatonin and ascorbic acid + N-acetyl cysteine reduced these levels. Ascorbic acid + N-acetyl cysteine caused a significant decrease in ALT level (*P* = 0.01). Additional administration of melatonin and ascorbic acid + N-acetyl cysteine resulted in a significant decrease in both total and direct bilirubin (*P* = 0.001).

DISCUSSION

The present study was undertaken to confirm the role of reactive oxygen species (ROS) in the pathogenesis

Table 4 AST, ALT, LDH, total and direct bilirubin levels in different groups after treatment (mean ± SE)

| Group | AST (U/L) | ALT (U/L) | LDH (U/L) | Total bilirubin (mg/dL) | Direct bilirubin (mg/dL) |
|---------------------------------|---------------------------|-------------------------|----------------------------|-------------------------|--------------------------|
| Control | 186.85±19.5 | 53.71±2.39 | 4088.43±510.5 | 0.02±0.02 | 0.01±0.01 |
| Caerulein | 259.57±18.60 ^a | 68.14±4.04 ^a | 7631.14±281.3 ^c | 6.88±0.12 ^c | 3.06±0.09 ^c |
| Caerulein + mel | 193.28±26.64 | 47.71±6.98 | 5065.71±508.10 | 0.04±0.01 ^e | 0.00±0.01 ^e |
| Caerulein + antioxidant mixture | 274.71±17.16 | 70.14±4.84 ^e | 6592.71±1065.41 | 0.05±0.02 ^e | 0.04±0.02 ^e |

^aP < 0.05 vs control; ^cP < 0.005 vs control; ^eP < 0.05 vs caerulein+mel; ^eP < 0.005 vs caerulein.

Table 5 Serum amylase and lipase levels in different groups after treatment (mean ± SE)

| Group | Amylase (U/L) | Lipase (U/L) |
|---------------------------------|-----------------------------|-------------------------|
| Control | 379.28±15.71 | 22.71±0.68 |
| Caerulein | 2182.29±563.92 ^a | 117.0±7.56 ^c |
| Caerulein + mel | 1406.71±169.44 | 65.0±10.81 |
| Caerulein + antioxidant mixture | 1881.57±414.20 | 70.71±8.72 |

^aP < 0.05 vs control; ^cP < 0.005 vs control.

of caerulein-induced acute pancreatitis and pancreatitis-induced hepatic damage. The exact pathogenesis of AP is not fully understood, but a large body of evidence suggests that pancreatic damage depends upon the toxic effect of ROS^[18,27]. However, few data are available regarding AP-induced hepatic oxidative stress^[19].

It is shown that acinar cells produce large amounts of ROS at early stage of AP in rats^[17]. Highly reactive ROS directly attacks lipids, proteins in the biological membranes and cause their dysfunction^[28]. Under normal conditions, a natural system of scavengers and antioxidants counteracts the cytotoxicity of ROS produced from molecular oxygen in the mitochondria. When the production of ROS is increased in AP by activated leukocytes, the capacity of intrinsic defense mechanism leads to alteration in cytoskeleton of acinar cells and damage of cell membranes^[27]. Degradation of polyunsaturated fatty acids in cell membranes by ROS results in the destruction of membranes and formation of MDA, which is an indicator of ROS generation^[29]. Disruption of the cytoskeleton leads to disturbance of intracellular transport of digestive enzymes and their premature activation and damage in acinar cells^[30]. Large amounts of ROS and activated pancreatic enzymes leaked from the broken cells injure capillary endothelium. Increased capillary permeability contributes to edema^[27]. It is suggested that PPAA contributes to hepatocyte injury by inducing hepatocyte apoptosis during AP. Barlas *et al*^[19] reported that ROS plays an import role in the pathogenesis of pancreatitis-induced hepatic damage.

In this study we measured the levels of MDA, a product of lipid peroxidation, and endogenous scavengers CAT and GPx in pancreatic and hepatic tissues. Caerulein-induced pancreatitis and liver damage were accompanied with a significant increase in tissue MDA levels ($P < 0.05$)

whereas a significant decrease in CAT and GPx activities ($P < 0.05$). These findings demonstrate that lipid peroxidation is not only dramatically enhanced within pancreatic tissue but also within hepatic tissue. Elevated MDA as an offense system and lowered CAT and GPx activities as a defense system play an important role in the development of pancreatic and liver injury.

Previous reports indicate that treatment with antioxidant agents such as melatonin^[19,20,22], N-acetylcysteine, ascorbic acid^[21], SOD, CAT and deferoxamine^[31], has beneficial effects on caerulein-induced AP. Melatonin can directly neutralize a number of free radicals and reactive oxygen and nitrogen species^[32-34] and stimulate several antioxidative enzymes (superoxide dismutase, glutathione peroxidase)^[32]. Free radical scavengers and antioxidants neutralize and/or metabolically remove reactive species from cells before they carry out their destructive activities^[33]. While the antioxidative actions of most molecules are limited by their specific intercellular distribution, antioxidative actions of melatonin include the protection of lipids in cell membranes, proteins in cytosol and DNA in nuclei. Furthermore, melatonin can crossover all morphophysiological barriers and enter all cells in the organism^[35]. In the present study, treatment with melatonin and N-acetylcysteine + ascorbic acid mixture significantly decreased histopathological damage score in both pancreas and liver. Additionally these therapies decreased tissue MDA levels but increased tissue CAT and GPx activities. Histopathological changes correlated with biochemical changes.

In conclusion, oxidative injury plays an important role not only in the pathogenesis of AP but also in pancreatitis-induced hepatic damage. Antioxidant agents such as melatonin and ascorbic acid + N-acetyl cysteine, are capable of limiting pancreatic and hepatic damage produced during AP *via* restoring tissue antioxidant enzyme activities.

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Ameliorative effect of *Ganoderma lucidum* on carbon tetrachloride-induced liver fibrosis in rats

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Abstract

AIM: To investigate the effects of *Reishi mushroom*, *Ganoderma lucidum* extract (GLE), on liver fibrosis induced by carbon tetrachloride (CCl₄) in rats.

METHODS: Rat hepatic fibrosis was induced by CCl₄. Forty Wistar rats were divided randomly into 4 groups: control, CCl₄, and two GLE groups. Except for rats in control group, all rats were administered orally with CCl₄ (20%, 0.2 mL/100 g body weight) twice a week for 8 weeks. Rats in GLE groups were treated daily with GLE (1 600 or 600 mg/kg) via gastrogavage throughout the whole experimental period. Liver function parameters, such as ALT, AST, albumin, and albumin/globulin (A/G) ratio, spleen weight and hepatic amounts of protein, malondialdehyde (MDA) and hydroxyproline (HP) were determined. Histochemical staining of Sirius red was performed. Expression of transforming growth factor β 1 (TGF- β 1), methionine adenosyltransferase (MAT1) 1A and MAT2A mRNA were detected by using RT-PCR.

RESULTS: CCl₄ caused liver fibrosis, featuring increase in plasma transaminases, hepatic MDA and HP contents, and spleen weight; and decrease in plasma albumin, A/G ratio and hepatic protein level. Compared with CCl₄ group, GLE (600, 1 600 mg/kg) treatment significantly increased plasma albumin level and A/G ratio ($P < 0.05$) and reduced the hepatic HP content ($P < 0.01$). GLE (1 600 mg/kg) treatment markedly decreased the activities of transaminases ($P < 0.05$), spleen weight ($P < 0.05$) and hepatic MDA content ($P < 0.05$); but increased hepatic protein level ($P < 0.05$). Liver histology in the GLE (1 600 mg/kg)-treated rats was also improved ($P < 0.01$). RT-PCR analysis showed that GLE treatment decreased the expression of TGF- β 1 ($P < 0.05-0.001$) and changed the expression of MAT1A ($P < 0.05-0.01$) and MAT2A ($P < 0.05-0.001$).

CONCLUSION: Oral administration of GLE significantly reduces CCl₄-induced hepatic fibrosis in rats, probably

by exerting a protective effect against hepatocellular necrosis by its free-radical scavenging ability.

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Key words: *Ganoderma lucidum*; Carbon tetrachloride; Liver fibrosis

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INTRODUCTION

Reishi mushroom, *Ganoderma lucidum* (Fr.) Krast (Polyporaceae), is a well-known Chinese crude drug used clinically in East Asia. The fruit bodies are used for the treatment of neurasthenia, deficiency fatigue, insomnia, bronchial cough in elderly people and carcinoma^[1].

A number of animal studies have indicated that water or ethanol extracts of *G. lucidum* showed protective actions against acute hepatitis in rats or mice^[2-4]. Other reports had previously indicated that triterpenoids isolated from *G. lucidum* possessed the protective effect against acute hepatitis caused by CCl₄^[5,6]. Furthermore, Park et al.^[7] demonstrated that, in rats, polysaccharides extracted from *G. lucidum* could antagonize liver fibrosis caused by biliary obstruction. These results demonstrate that *G. lucidum* possesses a protective effect in the liver.

Liver fibrosis is the common end-stage of most chronic liver disease, regardless of etiology, and its progression leads to cirrhosis and liver cancer^[8]. Although the exact mechanisms of pathogenesis in liver cirrhosis are still obscure, the role of free radicals and lipid peroxides has attracted considerable attention^[9]. It has been found that metabolism of CCl₄ involves the production of free radicals through its activation by drug-metabolizing enzymes located in the endoplasmic reticulum^[10]. CCl₄ is capable of causing liver lipid peroxidation, resulting in liver fibrosis^[11].

Data from *in vitro* and *in vivo* studies have indicated that *G. lucidum* has potent antioxidative and radical-scavenging effects^[3,12-15], which contribute to hepatoprotection^[3,15]. Nevertheless, to our knowledge, no reports have recorded the effect of *G. lucidum* on chronic hepatitis. In the present study, we therefore investigated the effect of extracts

of *G. lucidum* on chronic CCl₄-induced liver fibrosis.

MATERIALS AND METHODS

Preparation of test substance

Crude *G. lucidum* extract (GLE), which also contains cracked spores of *G. lucidum*, was obtained from the Taiwan branch of the American company NuSkin Pharmanex. GLE was suspended in distilled water and administered orally to each rat at a volume of 1 mL/100 g body weight. To guarantee reproducibility of pharmacological experiments, we assayed the total triterpene content of GLE.

Determination of total triterpenes in GLE by HPLC

GLE (100 mg) was extracted with ethyl acetate and then evaporated to dryness under vacuum. The residue was dissolved in methanol and diluted to 2 mL. The sample solutions were filtered through a 0.45- μ m filter before HPLC analysis as follows. HPLC instrument: Waters 2690 separation unit plus Waters 996 PDA; column: Phenomenex Lunca C18(2); flow rate: 1.0 mL/min; detection: absorption at 252 nm; gradient solvent system: CH₃CN+0.1% trifluoroacetic acid. The total peak area for a retention time of 8.0-38.0 min was used to calculate total triterpenes. The peak area of ganoderic acid A (Shanghai R&D, Pharmanex) was used as standard. This method showed that the total triterpene content of GLE was over 6%.

Animals

Male Wistar rats were obtained from the National Laboratory Animal Breeding and Research Center, National Science Council, and fed with a standard laboratory diet and tap water *ad libitum*. Experimental animals were housed in an air-conditioned room at 22-25°C and a 12 h light/dark cycle. Rats were allowed free access to powdered feed and mains water that was supplied through an automatic watering system. When they reached 250-300 g, forty rats were divided randomly into 4 groups, such as control, model and two GLE treatment groups, according to body weight 1 d before administration of the test substance. All animals received humane care and the study protocols were in compliance with Institutional Guidelines for the use of laboratory animals.

CCl₄-induced liver fibrosis

Liver fibrosis was induced by oral administration of 0.2 mL/100 g body weight of CCl₄ (200 mL/L; diluted in olive oil) twice a week for 8 wk. Animals received CCl₄ only (model group), CCl₄ with GLE (600 or 1 600 mg/kg per day) throughout whole experimental period. During CCl₄ administration, the time interval between CCl₄ and GLE administration was at least 5 h to avoid disturbance in absorption of each substance. At the end of the experimental period, rats were sacrificed under ether anesthesia and blood was withdrawn from the abdominal artery. Liver and spleen were quickly removed, weighed after washing with cold normal saline and removing excess moisture. The largest lobe of liver was divided into four parts, which were then used as follows: (1) submerged

in 100 mL/L neutral formalin for the preparation of pathological sections; (2) frozen directly in liquid nitrogen for transcript analysis; (3) after weighing, the liver was completely dried at 100 °C for the determination of collagen content; and (4) remaining samples were stored at -80 °C as reserves.

Assessment of liver functions

Whole blood was centrifuged at 4 700 r/min at 4 °C for 10 min to separate the plasma. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT), albumin and total protein were determined spectrophotometrically with an automatic analyzer (Cobas Mira; Roche, Rotkreuz, Switzerland) using commercially available kits (Roche Diagnostics).

Assays for hepatic protein, lipid peroxidation and hydroxyproline

Livers were homogenized in 9 vols ice-cold 0.15 mol/L KCl and 1.9 mmol/L ethylenediaminetetraacetic acid. Liver protein concentration was measured according to Lowry *et al*^[16] using bovine serum albumin as standard. Lipid peroxidation was measured by the methods of Ohkawa *et al*^[17] using 2-thiobarbituric acid. Lipid peroxidation was expressed as the amount of malondialdehyde/mg protein.

Hydroxyproline determination followed a method designed by Neuman and Logan^[18]. After hydrolysis, dried liver tissue was oxidized by H₂O₂ and colored by p-dimethylaminobenzoaldehyde; and absorbance was determined at 540 nm. The amount of hydroxyproline was expressed as μ g/g tissue.

RNA extraction and RT-PCR analysis

Total RNA was isolated from rat livers using the acid guanidium thiocyanate-phenol-chloroform extraction methods, as described by Chomczynski and Sacchi^[19]. A total of 5 μ g RNA from each liver sample was subjected to reverse transcription (RT) by using MMuLV reverse transcriptase in a 50 μ L reaction volume. Aliquots of the reverse transcription mixture were used for amplification by polymerase chain reaction (PCR) of fragments specific to transforming growth factor- β 1 (TGF- β 1), methionine adenosyltransferase 1A (MAT1A) and MAT2A using the primer pairs listed in (Table 1). The levels of expression of all transcripts were normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA in the same tissue sample. The primer pairs for TGF- β 1 and GAPDH were designed by Wolf *et al*^[20]. In addition, the primer pairs for MAT1A and MATA2A were designed using the Primer select program^[21]. The identities of the resultant PCR products were confirmed by sequence analysis. The cycling parameters were 30 min at 55°C for cDNA first strand synthesis, and 5 min at 95°C, 1 min at 55°C and 1 min at 72°C for 32 cycles in a Perkin Elmer 9700 Gene Amp PCR system. The PCR product was electrophoresed on a 20 g/L agarose gel recorded by Polaroid film, and the bands were quantitated by using densitometry.

Pathological examinations

After formalin fixation, tissue samples were sliced, embedded in a standard manner and stained with Sirius

Table 1 Primer sequences for PCR amplification

| mRNA | Primer sequences | Length (bp) |
|--------|---|-------------|
| TGF-β1 | Sense 5' TAT AGC AAC AAT TCC TGG CG 3' | 162 |
| | Antisense 5' TGC TGT CAC AGG AGC AGTG 3' | |
| MAT1A | Sense 5' AAA TGA AGA GGA TGT TGG TG 3' | 264 |
| | Antisense 5' ATT GTG TTG GCA CAG AGA GAT GA 3' | |
| MAT2A | Sense 5' ATG CTG TCC TTG ATG CAC 3' | 400 |
| | Antisense 5' GCG TA A CCA AGG CAA TG 3' | |
| GAPDH | Sense 5' CTT CAT TGA CCT CAA CTA CAT GGT CTA 3' | 99 |
| | Antisense 5' GATG ACA AGC TTC CCA TTC TCA G 3' | |

Table 3 Effect of GLE on plasma AST and ALT activity in CCl₄-treated rats

| Drugs | Dose (mg/kg) | AST (U/L) | ALT (U/L) |
|-------------------------------------|--------------|----------------------------|----------------------------|
| Control | - | 71.5 ± 15.6 | 41.9 ± 1.9 |
| CCl ₄ + H ₂ O | - | 610.8 ± 149.9 ^b | 464.7 ± 126.7 ^b |
| CCl ₄ + GLE | 600 | 648.9 ± 153.8 | 499.9 ± 112.1 |
| | 1600 | 459.3 ± 105.3 ^a | 324.7 ± 41.3 ^a |

^a*P* < 0.05 vs CCl₄ + H₂O group; ^b*P* < 0.001 vs control group.

red. Fibrosis was graded according to the method of Ruward *et al.*^[22] as follows: Grade 0 = normal liver; grade 1 = increase of collagen without formation of septa; grade 2 = formation of incomplete septa from portal tract to central vein (septa that do not interconnect with each other); grade 3 = complete but thin septa interconnecting with each other, so as to divide the parenchyma into separate fragments; and grade 4 = as grade 3, except with thick septa (complete cirrhosis). To avoid sampling error, all biopsies were obtained from the same lobe and these semi-quantitative grades were performed by the observer without knowledge of sample treatment.

Statistical analysis

Data were presented as mean ± SD. All other experimental data, except the pathological findings, were analyzed by one-way analysis of variance using the Dunnett's test. Liver histopathological examination data were analyzed by the Kruskal-Wallis non-parametric test, followed by a Mann-Whitney *U*-test. A *P* value < 0.05 was considered statistically significant.

RESULTS

Body weight and weights of liver and spleen

Treatment with CCl₄ caused a significant decrease in the body weight of rats as compared with control rats. There were no differences in the body weight of rats in the CCl₄ alone and CCl₄ + GLE groups. The final body weights for control and CCl₄-treated groups were 474.5 ± 26.7 and 405.4 ± 31.6 g, respectively.

CCl₄ treatment obviously caused splenomegaly in the rats, demonstrating that the weight of spleen in the CCl₄-treated group was about 290% of the control group (Table 2). On contrary, GLE (1 600 mg/kg) significantly reduced the weight of spleen induced by CCl₄ (Table 2). Moreover,

Table 2 Effect of GLE on weight of liver and spleen in CCl₄-treated rats

| Group | Dose (mg/kg) | Liver (g) | Spleen (g) |
|-------------------------------------|--------------|------------|--------------------------|
| Control | - | 15.1 ± 1.8 | 0.90 ± 0.11 |
| CCl ₄ + H ₂ O | - | 16.6 ± 3.3 | 2.58 ± 0.32 ^b |
| CCl ₄ + GLE | 600 | 16.7 ± 2.2 | 2.21 ± 0.36 |
| | 1600 | 18.2 ± 2.0 | 2.09 ± 0.56 ^a |

^a*P* < 0.05 vs CCl₄ + H₂O group; ^b*P* < 0.001 vs control group.

Table 4 Effect of GLE on plasma albumin concentration and A/G ratio in CCl₄-treated rats

| Group | Dose (mg/kg) | Albumin (g/dL) | A/G ratio |
|-------------------------------------|--------------|--------------------------|--------------------------|
| Control | - | 3.35 ± 0.15 | 1.22 ± 0.07 |
| CCl ₄ + H ₂ O | - | 2.33 ± 0.21 ^b | 0.75 ± 0.04 ^b |
| CCl ₄ + GLE | 600 | 2.73 ± 0.18 ^a | 0.87 ± 0.07 ^a |
| | 1600 | 2.70 ± 0.25 ^a | 0.88 ± 0.13 ^a |

^a*P* < 0.05 vs CCl₄ + H₂O group; ^b*P* < 0.001 vs control group.

CCl₄ did not change liver weights, and no effect of GLE on the liver weight was observed.

Effects of GLE on biochemical parameters

As shown in (Table 3), CCl₄ treatment resulted in a significant increase in plasma AST and ALT activities as compared to the control group. Oral administration of GLE (1 600 mg/kg) significantly reduced the CCl₄-induced increase in AST and ALT activities.

The plasma albumin content and A/G ratio in CCl₄-treated groups were significantly lower than that in the control group. The CCl₄-induced decrease in plasma albumin concentration and A/G ratio were significantly increased following the administration of GLE (600 and 1 600 mg/kg; Table 4).

Hepatic protein, malondialdehyde and hydroxyproline concentrations

CCl₄-induced liver fibrosis in rats resulted in a significant decrease in hepatic protein content compared to the control group. GLE (1 600 mg/kg) attenuated the decrease of hepatic protein level induced by CCl₄ (Table 5).

CCl₄ induced liver fibrosis in the rats, accompanied by a marked elevation of malondialdehyde and hydroxyproline concentrations. GLE (600 and/or 1 600 mg/kg) could lower the increase in hepatic malondialdehyde and hydroxyproline content (Table 5).

TGF-β1, MAT1A and MAT2A mRNA expression

Fragments specific to TGF-β1, MAT1A and MAT2A were amplified by using RT-PCR (Figure 1). Values from densitometric analysis, after normalization against the corresponding GAPDH transcript, were expressed as the TGF-β1:GAPDH, MAT1A:GAPDH and MAT2A:GAPDH ratios (Table 6). CCl₄ treatment could significantly increase the levels of both TGF-β1 and MAT2A. The administration of GLE (600 or 1600 mg/kg) significantly decreased the expression of TGF-β1 and

Table 5 Effect of GLE on hepatic protein, malondialdehyde and hydroxyproline content in CCl₄-treated rats

| Group | Dose (mg/kg) | Protein (mg/g tissue) | Malondialdehyde (nmol/mg protein) | Hydroxyproline (μg/g tissue) |
|-------------------------------------|--------------|---------------------------|-----------------------------------|------------------------------|
| Control | - | 186.3 ± 26.4 | 2.6 ± 0.5 | 550.0 ± 42.5 |
| CCl ₄ + H ₂ O | - | 108.7 ± 4.8 ^d | 4.4 ± 1.1 ^d | 1201.2 ± 151.7 ^d |
| CCl ₄ + GLE | 600 | 124.4 ± 10.2 | 3.8 ± 0.4 | 1050.4 ± 187.2 ^b |
| | 1600 | 128.2 ± 13.5 ^a | 3.4 ± 0.5 ^a | 877.5 ± 137.9 ^b |

^aP<0.05, ^bP<0.01 vs CCl₄ + H₂O group; ^dP<0.001 vs control group.

Table 6 Effect of GLE on the mRNA expression of hepatic TGF-β1, MAT1A and MAT2A

| Group | Dose (mg/kg) | TGF-β1/GAP-DH ratio | MAT1A/GAP-DH ratio | MAT2A/GAP-DH ratio |
|-------------------------------------|--------------|---------------------------|--------------------------|--------------------------|
| Control | - | 0.18 ± 0.03 | 4.34 ± 0.081 | 0.29 ± 0.02 |
| CCl ₄ + H ₂ O | - | 22.17 ± 7.20 ^f | 1.75 ± 0.46 ^f | 4.95 ± 0.21 ^f |
| CCl ₄ + GLE | 600 | 16.99 ± 3.26 ^a | 3.07 ± 1.15 ^a | 1.68 ± 0.14 ^d |
| | 1600 | 4.83 ± 0.48 ^d | 3.83 ± 1.35 ^b | 0.56 ± 0.03 ^d |

^aP<0.05, ^bP<0.01, ^dP<0.001 vs CCl₄ + H₂O group; ^fP<0.001 vs control group.

MAT2A mRNA. In contrast, the level of MAT1A mRNA was significantly decreased by CCl₄ treatment. However, treatment with GLE significantly increased the level of MAT1A mRNA.

Pathological changes

As shown in Figure 2, CCl₄ induced liver lesions in rats. Sirius red stain showed clear nodular fibrosis (Figure 2B). Treatment with GLE (1 600 mg/kg) showed a marked improvement in the pathological changes to these tissues (Figure 2C and Table 7).

DISCUSSION

The present study revealed the beneficial effect of GLE in prevention of liver fibrosis induced by CCl₄ treatment. An improvement brought about by GLE was also seen in plasma biochemical parameters.

CCl₄ treatment caused hepatocellular damage in rats, as indicated by a drastic increase in both plasma ALT and AST levels after CCl₄ administration. Rats treated with GLE showed a protection against CCl₄-induced hepatotoxicity, with the levels of both plasma AST and ALT being reduced.

It is well known that adenosylmethionine-dependent methylation is central to many biological processes^[23]. Methionine adenosyltransferase (MAT) is a key enzyme for liver methionine metabolism, which catalyzes the synthesis of S-adenosylmethionine^[24]. In mammalian tissue, three different forms of MAT (MAT I/III and MAT II) have been identified, which are the product of two different genes (MAT1A and MAT2A). MAT1A is primarily restricted to adult liver^[25]. MAT2A is high in fetal liver,

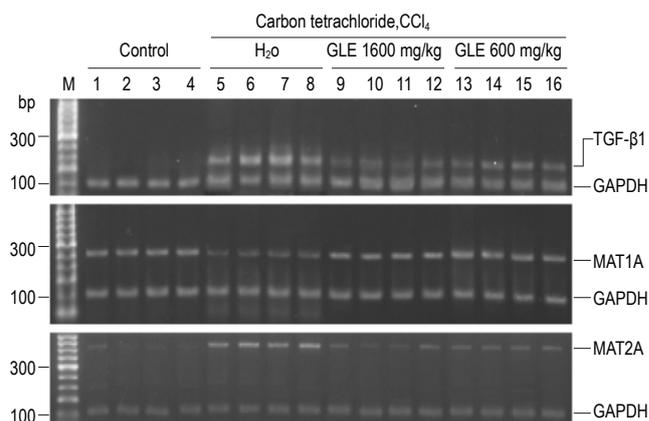


Figure 1 Effect of GLE on mRNA expression of TGF-β1, MAT1A and MAT2A in hepatic tissue. M: DNA marker

decays at birth to negligible levels and, in the adult liver, increases during regeneration after partial hepatectomy^[26-28]. Thus, in response to liver injury, MAT1A expression is switched off and MAT2A expression is switched on. Consistent with this, the expression of MAT1A was found to be reduced in the livers of rats with chronic CCl₄ injury, whereas the expression of MAT2A increased. In this study, we also found that the changes in MAT expression in chronic CCl₄-injured rats were reduced by GLE treatment. These results further support the fact that GLE possesses a hepatoprotective effect.

The liver synthesizes not only the protein it needs, but also produces numerous export proteins. Among the latter, plasma albumin is the most important^[29]. Export proteins are synthesized on polyribosomes bound to the rough endoplasmic reticulum of the hepatocytes. In contrast, protein destined for intracellular use is synthesized on free polyribosomes rather than bound polyribosomes^[29]. In this experiment, CCl₄ induced liver fibrosis in rats and it appeared to cause a decrease in both hepatic protein and plasma albumin contents. GLE clearly reduced the decrease in protein content in the liver and albumin content in the plasma; thus it was shown to ameliorate the decline in liver synthesis function caused by CCl₄-induced fibrosis.

Immunoglobulin is synthesized by immunocytes and hyperglobulinemia is found in hepatocellular disorders, appearing as an inflammatory reaction of liver^[30]. In the present experiments, we observed CCl₄-induced chronic liver lesions in rats and also a decrease in A/G ratio. GLE could clearly lessen the decrease in the A/G ratio caused by CCl₄, thereby exhibiting suppressive actions on liver

Table 7 Effect of GLE on CCl₄-induced liver fibrosis in rats

| Group | Dose (mg/kg) | Score of hepatic fibrosis | | | | | Average |
|-------------------------------------|--------------|---------------------------|---|---|---|---|----------------------|
| | | 0 | 1 | 2 | 3 | 4 | |
| Control | - | 10 | 0 | 0 | 0 | 0 | 0 |
| CCl ₄ + H ₂ O | - | 0 | 0 | 1 | 9 | 0 | 2.9±0.3 |
| CCl ₄ + GLE | 600 | 0 | 0 | 3 | 7 | 0 | 2.7±0.3 |
| | 1600 | 0 | 0 | 6 | 4 | 0 | 2.4±0.6 ^b |

^bP<0.01 vs CCl₄ group.

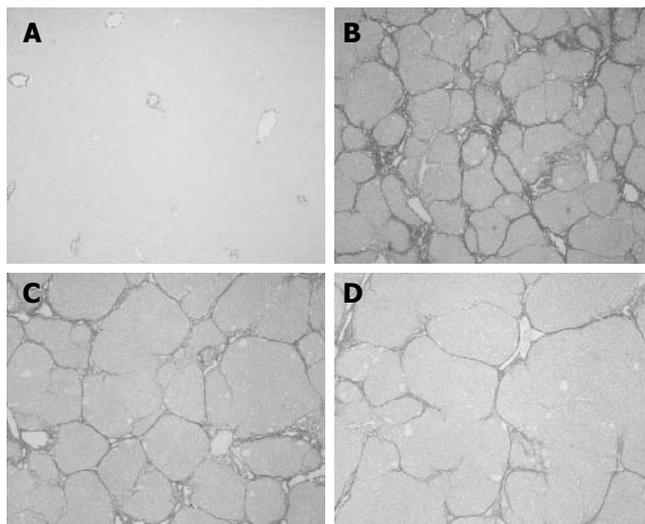


Figure 2 Sirius red staining of rat liver sections. **A:** Control; **B:** CCl₄+H₂O, showing micronodular formation and complete septa interconnection with each other; **C:** CCl₄+GLE (600 mg/kg); **D:** CCl₄+GLE (1600 mg/kg), showing a marked reduction in fiber deposition.

inflammation caused by CCl₄.

It is well known that liver fibrosis is a result of increased collagen synthesis^[31]; hydroxyproline is the characteristic component in collagen^[32]. The amount of collagen can be reflected by determining hydroxyproline concentration and can be used to express the extent of fibrosis^[32]. In this experiment, when CCl₄ was administered to induce liver fibrosis, the hydroxyproline contents in liver obviously increased. Interestingly, GLE could reduce hydroxyproline concentration, indicating that it could lessen the actions of hepatic fibrosis caused by CCl₄, which was further proved by histopathological inspection.

When the liver is damaged, it can initiate regenerative actions^[33], thus increasing the weight of liver. If it was heavily damaged, however, liver fibrosis and cirrhosis appear resulting in liver atrophy^[34]. Therefore, the change in weight of liver can not directly predict the pathological process in chronic liver injuries.

Liver fibrosis leads to blockage of blood flow into the liver and causes portal hypertension. It also influences blood flow to the spleen and gives rise to splenomegaly^[35]. In this study, CCl₄ induced chronic hepatic fibrosis and splenomegaly, but GLE could improve splenomegaly, indicating that GLE possesses actions in ameliorating fibrosis.

Increased free radical production and lipid peroxidation

have been proposed as a major cellular mechanism involved in CCl₄ hepatotoxicity^[10]. Furthermore, a close relationship has been reported between lipid peroxidation and fibrogenesis in rats, in which fibrosis was induced by CCl₄ administration^[11]. Our results also confirmed these findings that hepatic lipid peroxidation increases during hepatic fibrogenesis. Moreover, we observed that GLE inhibited CCl₄-induced hepatic lipid peroxidation. These results indicated that GLE might inhibit lipid peroxidation and consequently attenuate the development of liver fibrosis. Numerous studies have indicated that *G. lucidum* extracts are good free-radical scavengers^[12-14], suggesting that the ameliorative effects of GLE on liver fibrosis induced by CCl₄ are due, at least in part, to its free-radical scavenging ability.

TGF-β1 is a profibrogenic cytokine as it directly stimulates extracellular matrix production by both endothelial and stellate cells^[36,37]. Increased levels of TGF-β1 mRNA expression have been found in patients with liver fibrosis as well as in experimental models of liver fibrosis^[38,39]. Blockade of TGF-β1 synthesis or signaling is a primary target for the development of antifibrotic approaches, and modern hepatology has facilitated the design of drugs removing this causative agent^[40]. In this study, CCl₄ treatment increased while GLE treatment significantly reduced TGF-β1 mRNA expression, suggesting that GLE might ameliorate liver fibrosis via reducing TGF-β1 secretion.

In conclusion, oral administration of GLE is effective in the reduction of chronic liver injury, probably via a protective effect against hepatocellular necrosis by its free-radical scavenging ability.

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Systemic phosphatidylcholine pretreatment protects canine esophageal mucosa during acute experimental biliary reflux

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Abstract

AIM: To characterize the consequences of short-term exposure to luminal bile on mucosal mast cell reactions in a canine model, and to determine the effects of systemic phosphatidylcholine pretreatment in this condition.

METHODS: Twenty mongrel dogs were used for experiments. Group 1 ($n=5$) served as a saline-treated control, while in group 2 ($n=5$) the esophagus was exposed to bile for 3 h. In group 3 ($n=5$) the animals were pretreated with 7-nitroindazole to inhibit the neuronal isoform of nitric oxide synthase. In group 4 ($n=5$) phosphatidylcholine solution (50 mg/kg) was administered iv before the biliary challenge. Mucosal microcirculation was observed by intravital videomicroscopy. Myeloperoxidase and nitric oxide synthase activities, the degrees of mast cell degranulation and mucosal damage were evaluated via tissue biopsies.

RESULTS: Exposure to bile evoked significant mast cell degranulation and leukocyte accumulation. The red blood cell velocity and the diameter of the postcapillary venules increased significantly. The tissue ATP content and constitutive nitric oxide synthase activity decreased, while the inducible nitric oxide synthase activity increased significantly as compared to the control values. 7-nitroindazole treatment significantly exacerbated the mucosal mast cell degranulation and tissue damage. In contrast, phosphatidylcholine pretreatment prevented the bile-induced ATP depletion, the inducible nitric oxide synthase and myeloperoxidase activity and the mast cell degranulation increased.

CONCLUSION: The neuronal nitric oxide synthase - mast cell axis plays an important role in the esophageal mucosal defense system. Systemic phosphatidylcholine pretreatment affords effective protection through ameliorating the bile-induced ATP depletion and

secondary inflammatory reaction.

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Key words: Esophagus; Bile; Neuronal nitric oxide; Mast cell; Microcirculation; Inflammation

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INTRODUCTION

During acute regurgitation or prolonged gastroesophageal reflux episodes, the esophageal epithelial layer is exposed to various noxious luminal agents. Gastric acid has been shown to play a crucial role in the development of esophagitis, but regurgitated bile could also be linked to various detrimental mucosal reactions, including ATP depletion and permeability alterations^[1,2]. Bile salts can damage the epithelium both directly and indirectly, and may alter the function of cells. Given that mast cells (MCs) are involved in stress-induced gastrointestinal reactions, it seems reasonable to assume that the effects of bile involve MCs. Indeed, it has been shown *in vitro* that bile acids induce concentration-dependent MC degranulation in correlation with their lipophilicity and surface activity^[3].

A number of MC-specific reactions in the gastrointestinal tract may be linked to the activity of constitutively expressed nitric oxide synthase (NOS). A relative lack of NO can activate both MCs and leukocytes, and MC degranulation *per se* can bring about leukocyte accumulation and other characteristics of local inflammation^[4,5]. NOS system consists of three distinct isoforms: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). Both eNOS and nNOS are constitutively expressed, but nNOS is predominant in the gastrointestinal tract^[6,7]. Although the above line of reasoning suggests that MCs and nNOS-derived NO could be closely associated with mucosal homeostasis, the relative contributions of the different NOS isoforms to the reflux-caused inflammatory responses of the esophagus are unclear. In the first part of our study, therefore, we examined the consequences of an acute

biliary challenge on NOS activation and mucosal MC reactions by performing detailed microcirculatory, histological and biochemical analyses. As we hypothesized that bile-induced inflammatory response critically involves NO, we determined the consequences of nNOS inhibition in this setting.

In addition to this pathomechanism investigation, another aim was to outline a means of modulating the outcome of biliary mucosal irritation. It is clearly recognized that a mixed or biliary reflux is more harmful than gastric acid alone, but to date there are no effective pharmacological therapies for bile-induced esophagitis. Nevertheless, various lines of indirect evidence have suggested that phosphatidylcholine (PC) may be protective during this condition. It was postulated in early *in vitro* studies that PC in aqueous media may protect against the membrane damage caused by bile salts^[8, 9]. In stress conditions the hydrolysis of endogenous membrane PC leads to release of phosphatidic acid and choline. Choline is anti-inflammatory and is actively transported into the epithelial cells^[10]. Moreover, choline could form part of a defense mechanism which may operate in biological systems against oxido-reductive stress^[11].

Accordingly, in the second part of our study we set out to establish whether systemic PC administration can protect the esophageal mucosa by acting as an anti-inflammatory agent in bile-induced esophagitis.

MATERIALS AND METHODS

The experiments were performed in adherence to the NIH guidelines for the use of experimental animals. The study was approved by the Ethical Committee for the Protection of Animals in Scientific Research at the University of Szeged.

Surgical preparation

The experiments were performed on 20 inbred mongrel dogs (average weight 12 ± 3 kg) under sodium pentobarbital anesthesia (30 mg/kg iv). Small supplementary doses of pentobarbital were administered when necessary. The left femoral artery and vein were cannulated for recording the mean arterial pressure and blood sampling, respectively. The animals were placed in a supine position on a heating pad for maintenance of the body temperature between 36°C and 37°C, and received an infusion of Ringer's lactate at a rate of 10 mL/(kg h) during the experiments.

Following a collar incision, the cervical esophagus with intact neurovascular connections was dissected free, and an approximately 8-10-cm segment of the middle portion was then occluded at both ends with atraumatic clips. The objective of the videomicroscope (Cytoscan A/R, Cytometrics, PA., USA) was placed into the esophagus lumen for continuous observation of the microcirculation. A second polyvinyl tube (0.5 mm i. d.) was secured with purse-string sutures for administration of the test compounds.

Experimental protocol

Surgery was followed by a 30-min recovery period for cardiovascular stabilization, and 7.0 mL isotonic saline

(pH 7.4) was then injected into the lumen of the dissected esophagus for 30 min in order to determine the baseline variables. The esophageal segment was next filled with test solution (7.0 mL) for 180 min. At the end of the experiment, a biopsy was taken from the esophageal segment, together with a tissue sample from the aboral intact part of the esophagus, with a freeze-clamp technique for determination of the tissue adenosine triphosphate (ATP) concentrations, and additional biopsies were performed to measure the tissue myeloperoxidase (MPO) and NOS isoform activities, and to determine the extent of MC degranulation and structural damage.

The animals were randomly allotted into 4 groups. Group 1 ($n=5$) served as saline-treated control, while in groups 2-4 the effects of bile were investigated. In group 2 ($n=5$) the animals were treated with canine bile alone. Bile obtained from 3 healthy dogs was pooled and stored at -20°C, and diluted freshly (pH 6.5) before the experiments. Bile was diluted with same volume of saline and solution was warmed up to 37°C before administration. Group 3 ($n=5$) was treated with 5 mg/kg 7-nitroindazole (7-NI, Sigma Chem., USA) in 0.3mL/min iv infusion 20 min before bile administration. In group 4 ($n=5$) the animals received 50 mg/kg iv PC infusion 20 min before bile treatment. Five percent PC solution (soybean lecithin, MW: 785, phospholipon 90, Phospholipid GmbH, Cologne, Germany) was freshly prepared according to the description of the manufacturer. Briefly, PC solution contained deoxycholate (2.3%), NaOH (0.24%), benzyl alcohol (0.82%), NaCl (0.22%), and ethanol (0.27%) in distilled water. The intraluminal volume load was identical in all groups studied.

Measurements

Central venous pressure and mean arterial pressure were measured continuously with Statham P23Db transducers and registered with a computerized data-acquisition system (Haemosys 1.17, Experimetria Ltd., Budapest, Hungary). Arterial blood gases were measured with a blood gas analyzer (AVL Compact 2, Graz, Austria).

Intravital video-microscopy

The intravital OPS technique (Cytoscan A/R, Cytometrics, PA, USA) was used for continuous visualization of the microcirculation of the esophageal mucosa. This technique utilizes reflected polarized light at the wavelength of the isosbestic point of oxy- and deoxyhemoglobin (548 nm). Since polarization is preserved in reflection, only photons scattered from a depth of 2-300 μ m contribute to the image formation. A 10x objective was introduced into the intestinal lumen, and the microscopic images were recorded with a S-VHS video recorder (Panasonic AG-TL 700). Videomicroscopic observations were made at specific anatomic locations at a depth of approximately 200 μ m. Microcirculatory evaluation was performed off-line by frame-to-frame analysis of the videotaped images. Capillary red blood cell velocity (RBCV, μ m/s), vessel diameter (VD, μ m) changes in postcapillary venules and relative vessel area (RVA, length of perfused nutritive capillaries per total capillary length of a standard observation area) were determined in 3 separate fields by

means of a computer-assisted image analysis system (IVM Pictron, Budapest, Hungary). Changes in venular wall diameter were determined by following identifiable visual landmarks within the vessel wall. All microcirculatory evaluations were performed by one investigator (EG).

ATP measurement

A whole-thickness sample was taken from the esophagus with a Wollenberg forceps cooled in liquid nitrogen, and the tissue was stored at -70°C . The sample was weighed, placed into a 3-fold volume of trichloroacetic acid (6% w/v), homogenized for 1 min, and centrifuged at 5000 g/min. The supernatant was neutralized with saturated potassium carbonate solution. The ATP concentration was measured spectrophotometrically according to Lamprecht *et al.*^[12]. The method is based on the principle that β -nicotinamide adenine dinucleotide phosphate is used up in an enzymatic reaction catalyzed by glucose-6-phosphate dehydrogenase and hexokinase.

NOS activity measurements

NO formation in esophageal tissue was measured by the conversion of [^3H] L-citrulline from [^3H] L-arginine according to the method of Szabo *et al.*^[13]. Briefly, tissue homogenates were centrifuged at 24 000 g/min for 20 min at 4°C and the supernatant was loaded into centrifugal concentrator tubes (Amicon Centricon-100; 100 000 MW cut-off ultrafilter). The tubes were centrifuged at 1000 g/min for 150 min and the concentrated supernatant was washed out from the ultrafilter with 300 μL homogenizing buffer. The samples were incubated with a cation-exchange resin (Dowex AG 50W-X8, Na^+ form) for 5 min to deplete endogenous L-arginine. The resin was separated by centrifugation at 1500 g/min for 10 min and the supernatant containing the enzyme was assayed for NOS activity. For the Ca^{2+} -dependent NOS (cNOS) activity, 50 μL enzyme extract and 100 μL reaction mixture (pH 7.4, containing 50 mmol/L Tris-HCl buffer, 1 mmol/L NADPH, 10 $\mu\text{mol/L}$ tetrahydrobiopterin, 1.5 mmol/L CaCl_2 , 100 U/mL calmodulin and 0.5 μCi [^3H] L-arginine (ICN Biomedicals, specific activity 39 Ci/mmol) were incubated together for 30 min at 37°C . The reaction was stopped by the addition of 1 mL ice-cold HEPES buffer (pH 5.5) containing 2 mmol/L EGTA and 2 mmol/L EDTA. Measurements were performed with boiled enzyme and the NOS inhibitor N- ω -nitro-L-arginine (3.2 mmol/L) to determine the extent of [^3H] L-citrulline formation that was independent of the NOS activity. Ca^{2+} -independent NOS activity (iNOS) was measured without Ca-calmodulin but with EGTA (8 mmol/L). Reaction mixture (1 mL) was applied to Dowex cation-exchange resin (AG 50W-X8, Na^+ form) and eluted with 2 mL distilled water. The eluted [^3H] L-citrulline activity was measured with a scintillation counter (Tri-Carb Liquid Scintillation Analyze 2100TR/2300TR, Packard Instrument Co, Meriden, Ct., USA) and referred to the protein content.

Tissue MPO activity

The activity of MPO, a marker of tissue leukocyte infiltration, was measured from mucosal biopsies by the method of Kuebler *et al.*^[14]. Briefly, the tissue was

homogenized with Tris-HCl buffer (0.1 mmol/L, pH 7.4) containing 0.1 mM polymethylsulfonyl fluoride to block tissue proteases, and then centrifuged at 2000 g/min for 20 min at 4°C . The MPO activities of the samples were measured at 450 nm (UV-1601 spectrophotometer, Shimadzu, Japan), and the data were referred to the protein content.

Light microscopy

Esophageal biopsy samples were rapidly placed into ice-cold Carnoy's fixative, embedded in paraffin, sectioned (6 μm) and stained with hematoxylin-eosin. Histological analysis was performed in coded sections by one investigator (MB). Mucosal injury was graded on the 0-100 esophageal mucosal damage score of Lanás *et al.*^[15] with the following criteria: epithelial changes (epithelial splitting, erosion and ulceration): maximal score 40; inflammation (intraepithelial leukocytes and cellular hyperplasia): maximal score 40; vascular lesions (edema, congestion and hemorrhage): maximal score 20.

Counting of polymorphonuclear leukocytes (PMN) was performed on coded sections by two independent investigators (TL, EG). Five nonoverlapping fields were observed in each section, and in each field an average of 4 consecutive measurements was used to calculate average PMN count with a semiquantitative scoring system. PMN counts were determined in the epithelium, submucosa, muscle layer and adventitia with the following criteria: grade 0, no PMNs in the given structure; grade 1, 1-10 PMNs; grade 2, 11-100 PMNs; grade 3: > 100 PMNs.

Histology for mast cells

The sections were stained with acidic toluidine blue (pH 0.5) and alcian blue-safranin O (Sigma Chem. USA, pH 0.4). Positively stained MCs were quantitated in 10 fields. The counting was performed in coded sections at an optical magnification of $\times 400$ by two independent investigators (EG and TL). Loss of intracellular granules and stained material dispersed diffusely within the lamina propria were taken as evidence of MC degranulation. For each animal, 5 fields were chosen at random, and the number of degranulated and intact MCs was counted. All counts were pooled for each treatment, and the percentage of degranulated MCs was calculated.

Statistical analysis

Data analysis was performed with a statistical software package (SigmaStat for Windows, Jandel Scientific, Erkrath, Germany). Nonparametric methods were used. Friedman repeated measure analysis of variance on ranks was applied within the groups. Time-dependent differences from the baseline were assessed by Dunn's method. Differences between groups were analyzed with Kruskal-Wallis one-way analysis of variance on ranks, followed by Dunn's method for pairwise multiple comparison. In the Table and Figures, median values and 75th and 25th percentiles are given. $P < 0.05$ was considered statistically significant.

RESULTS

The baseline values of the macrohemodynamic variables

Table 1 Effects of various intraluminal treatments on the mean arterial pressure (MAP), heart rate (HR) and red blood cell velocity (RBCV, $\mu\text{m/s}$) and venular diameter (VD, μm) of the esophageal mucosa.

| Group | Time | -30 min | 0 | 30 min | 60 min | 90 min | 120 min | 180 min |
|-------------------|----------|------------|------------|------------|------------|------------|------------|----------|
| Saline | RBCV(M) | 600 | 608 | 572 | 615 | 569 | 575 | 630 |
| | 25p; 75p | 554; 661 | 548; 663 | 536; 662 | 526; 709 | 539; 673 | 524; 705 | 540; 705 |
| | VD (M) | 31.0 | 33.0 | 33.0 | 32.0 | 33.0 | 33.0 | 34.0 |
| | 25p; 75p | 27.3; 34.3 | 27.5; 34.5 | 27.3; 34.0 | 28.3; 34.8 | 28.3; 34.0 | 27.5; 34.0 | 31; 34 |
| | MAP (M) | 163 | 161 | 158 | 157 | 150 | 156 | 152 |
| | 25p; 75p | 134.5; 172 | 132; 175 | 130; 168 | 132; 167 | 129; 169 | 128; 168 | 129; 169 |
| | HR (M) | 172.5 | 171 | 169 | 167 | 177 | 180 | 181 |
| | 25p; 75p | 126; 200 | 145; 183 | 151; 206 | 141.5; 198 | 142; 195 | 141; 194 | 146; 188 |
| | RBCV(M) | 621 | 662 | 717 | 783 a | 770 ac | 777 ac | 813 ac |
| Bile | 25p; 75p | 573; 660 | 574; 693 | 610; 791 | 734; 821 | 686; 828 | 712; 836 | 728; 869 |
| | VD (M) | 33.0 | 33.5 | 41 ac | 45.5 ac | 43.0 ac | 42.5 ac | 43.5 ac |
| | 25p; 75p | 33.0; 34.0 | 33; 35.0 | 39; 43.0 | 41.0; 48.0 | 41.0; 45.0 | 42.0; 44.0 | 42.0; 47 |
| | MAP (M) | 155 | 157 | 153 | 137 | 145 | 145 | 147 |
| | 25p; 75p | 129; 185 | 131; 181 | 128; 184 | 127; 185 | 126; 176 | 121; 181 | 127; 185 |
| | HR (M) | 167 | 171 | 161 | 161 | 181 | 177 | 192 |
| | 25p; 75p | 160; 174 | 157; 191 | 149; 171 | 158; 170.5 | 141.5; 197 | 154; 197 | 153; 194 |
| | RBCV(M) | 635 | 651 | 789 c | 809 ac | 821 ac | 784 ac | 818 ac |
| | 25p; 75p | 623; 651 | 640; 703 | 698; 872 | 694; 888 | 715; 881 | 727; 806 | 724; 870 |
| PC + bile | VD (M) | 36.0 | 35.0 | 37.0 ce | 37.5 ce | 39.0 ce | 38.0 ce | 38.0 ce |
| | 25p; 75p | 33.0; 38.5 | 32; 39.0 | 33.5; 40 | 35.5; 41.5 | 34.0; 41.0 | 33.0; 42.5 | 34.5; 42 |
| | MAP (M) | 153 | 147.5 | 161.5 | 152.5 | 150 | 162.5 | 160 |
| | 25p; 75p | 132.5; 164 | 130; 154 | 133; 170 | 131; 167.5 | 129; 168.5 | 132; 174 | 133; 173 |
| | HR (M) | 164 | 162 | 164 | 162.5 | 171 | 180 | 188 |
| | 25p; 75p | 154; 193 | 145; 167 | 143; 180 | 148; 188 | 142; 199 | 158; 186 | 183; 198 |
| | RBCV(M) | 625 | 632 | 791 c | 762 a | 821 ac | 793 c | 872 ac |
| | 25p; 75p | 556; 696 | 614; 671 | 649; 870 | 661; 893 | 761; 870 | 718; 845 | 857; 893 |
| | VD (M) | 31.0 | 32.0 | 34.0 e | 34.0 e | 32.5 e | 34.0 e | 33.5 e |
| 7-NI + bile | 25p; 75p | 30.0; 31.0 | 29; 33.0 | 33; 36 | 34.0; 34.0 | 31.0; 36.0 | 32.0; 34.0 | 32; 34.0 |
| | MAP (M) | 132.5 | 138 | 145 | 137.5 | 141.5 | 135.5 | 139.5 |
| | 25p; 75p | 128; 144 | 133; 150 | 132; 150 | 130; 151 | 134; 149 | 130; 143 | 135; 145 |
| | HR (M) | 170 | 163 | 171 | 165 | 181 | 185 | 186 |
| | 25p; 75p | 162; 180 | 159; 169 | 158; 182 | 152; 178 | 173; 191 | 176.5; 196 | 182; 189 |

M=median value, 25p and 75p=25th and 75th percentiles, respectively. ^a $P<0.05$ vs -30-min values, ^c $P<0.05$ vs saline-treated group, ^e $P<0.05$ vs bile-treated group.

did not differ significantly in the different groups and there were no significant hemodynamic changes as compared to the control values during the experimental period (Table 1). The mean arterial pressure in the bile+7-NI or PC-treated groups was not significantly different ($P>0.05$) from that in the saline-treated group as a whole (Table 1).

Venules of $35 \pm 10 \mu\text{m}$ in diameter were the largest fraction of the vessels and arterioles were seen only very rarely. The baseline level of RBCV in the various groups ranged 560 - 680 $\mu\text{m/s}$, and in the control group the RBCV did not change during the experiments (Table 1). However, the RBCV was increased significantly after 3-h exposure to bile with or without 7-NI or PC pretreatment. The median value was 813, 886 and 787 $\mu\text{m/s}$ after bile, bile with 7-NI, and bile with PC pretreatment, respectively.

The inner boundary of the venular wall was easily distinguishable and an exact comparison of inner diameter changes was possible. The median vessel diameter was significantly greater in bile-treated group 2 as compared to the saline-treated control, and increased from the baseline value of 33 μm to 44 μm . Administration of 7-NI before bile treatment prevented this increase. PC pretreatment resulted in a significant vessel diameter elevation as

compared to the control group, but the diameter changes in this group were significantly lower than those in the bile-treated group (Table 1).

The *in vivo* interference of bile treatment with ATP production of the esophageal mucosa was evaluated (Figure 1). There were no significant differences in tissue ATP levels between the intact and treated parts of the esophagus in the saline-treated control group (data not shown). There was a statistically significant fall (45%) in the ATP content of the esophageal tissue after bile treatment (saline: M=4.43 $\mu\text{mol/mg}$ protein, 25p=4.21, 75p=4.76; bile: M=2.42 $\mu\text{mol/mg}$ protein, 25p=1.66, 75p=2.61) by the end of the observation period, and this change was not affected by 7-NI treatment. Following PC treatment, the ATP content was significantly maintained in the mucosa as compared to bile treatment alone (M=3.82, 25p=3.56, 75p=4.28), and this value was not significantly different from the corresponding value in the saline-treated group.

Figure 2 demonstrates the changes in esophageal cNOS and iNOS activities. The activity of cNOS was significantly depressed after bile treatment, and this change was accompanied with a significant increase (6-fold) in

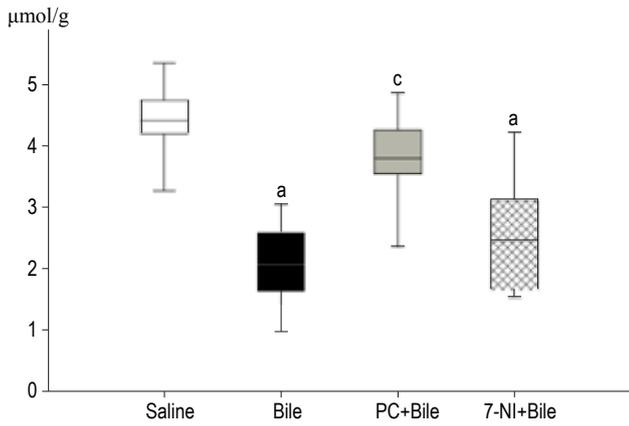


Figure 1 Effects of bile (black box), PC+bile (gray box) and 7-NI+bile (checked box) on the ATP content of esophageal mucosa. The plots demonstrate the median (horizontal line in the box), the 25th (lower whisker) and 75th (upper whisker) percentiles, respectively. ^a*P*<0.05 between groups vs saline-treated control group. ^c*P*<0.05 between bile+7-NI-treated and bile+PC-treated groups vs bile-treated group values.

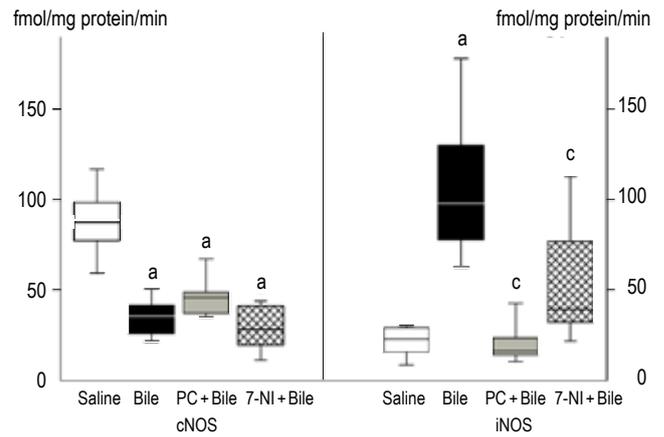


Figure 2 Changes in constitutive (left panel) and inducible NOS (right panel) activities [fmol (mg protein)⁻¹/min] in esophageal tissue from saline-treated (empty box), bile-treated (black box), bile+PC-treated (gray box) and bile+7-NI-treated (checked box) animals. The plots demonstrate the median (horizontal line in the box), the 25th (lower whisker) and 75th (upper whisker) percentiles, respectively. ^a*P*<0.05 between groups vs saline-treated control group values. ^c*P*<0.05 between bile+7-NI-treated and bile+PC-treated groups vs bile-treated group values.

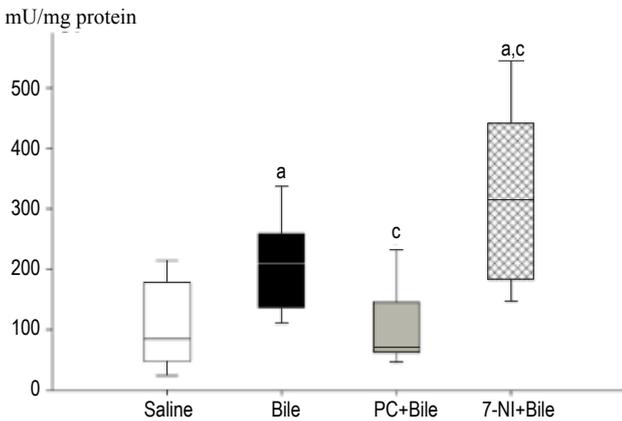


Figure 3 Myeloperoxidase (MPO) activity in the esophageal mucosa 180 min after treatment. The plots demonstrate the median (horizontal line in the box), the 25th (lower whisker) and 75th (upper whisker) percentiles, respectively. ^a*P*<0.05 between groups vs saline-treated control group values. ^c*P*<0.05 between bile+7-NI-treated and bile+PC-treated groups vs bile-treated group values.

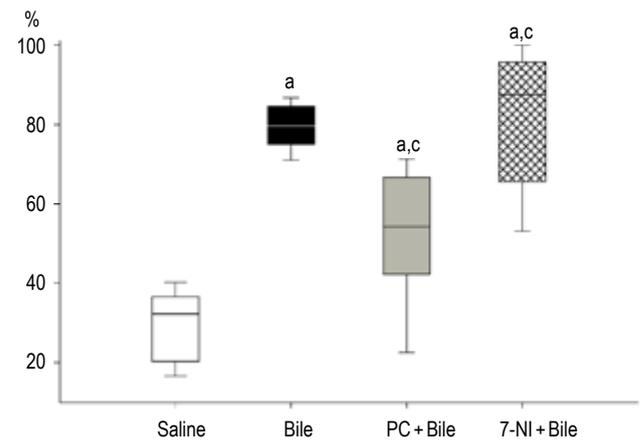


Figure 4 Mast cell degranulation (%) in the esophageal mucosa 180 min after treatment. The plots demonstrate the median (horizontal line in the box) and the 25th (lower whisker) and 75th (upper whisker) percentiles, respectively. ^a*P*<0.05 between groups vs saline-treated control group values. ^c*P*<0.05 between bile+7-NI-treated and bile+PC-treated groups vs bile-treated group values.

iNOS activity. The cNOS activity was not altered by PC or 7-NI pretreatment. However, the esophageal iNOS activity was significantly lower after PC pretreatment as compared to bile treatment alone. 7-NI treatment resulted in a somewhat lower increase (2.6-fold) in iNOS activity, as compared to the value in the bile-treated group.

The MPO data demonstrated that leukocyte accumulation was significantly increased in mucosae of the bile-treated and 7-NI pretreated groups as compared to the saline-treated group (Figure 3). Bile alone resulted in a 2.5-fold (M=209.4; 25p=136.6; 75p=259.6) rise in MPO activity, and a further increase was observed after 7-NI+bile administration (M=315.4, 25p=182.4, 75p=441.8). PC pretreatment significantly decreased bile-induced MPO activity.

Histological scoring of leukocyte infiltration was performed by two independent viewers (the inter-observer

variation was less than 15%, and these data showed good correlation with MPO results). In saline-treated group the extravasation of PMNs was negligible (range of scores: 1-3; M=2). Exposure to bile resulted in a significant (*P*<0.05) accumulation of PMNs (range: 4-6; M=5). In PC pretreated animals the PMN infiltration was significantly decreased and the scores did not differ significantly from the control values (range: 1-3; M=3). Bile + 7-NI treatment increased the degree of PMN infiltration and resulted in a grade 7 score (range: 7-9).

The effects of bile and various pretreatments on the mucosal MC degranulation are presented in Figure 4. In the control group, 35% of the MCs were degranulated. Exposure to bile resulted in a significant degranulation (M=79.6%, 25p=75.0, 75p=84.6) relative to the control group. In the PC-pretreated group, the percentage of degranulated MCs (M=54.3%, 25p=42.2%, 75p=66.7%)

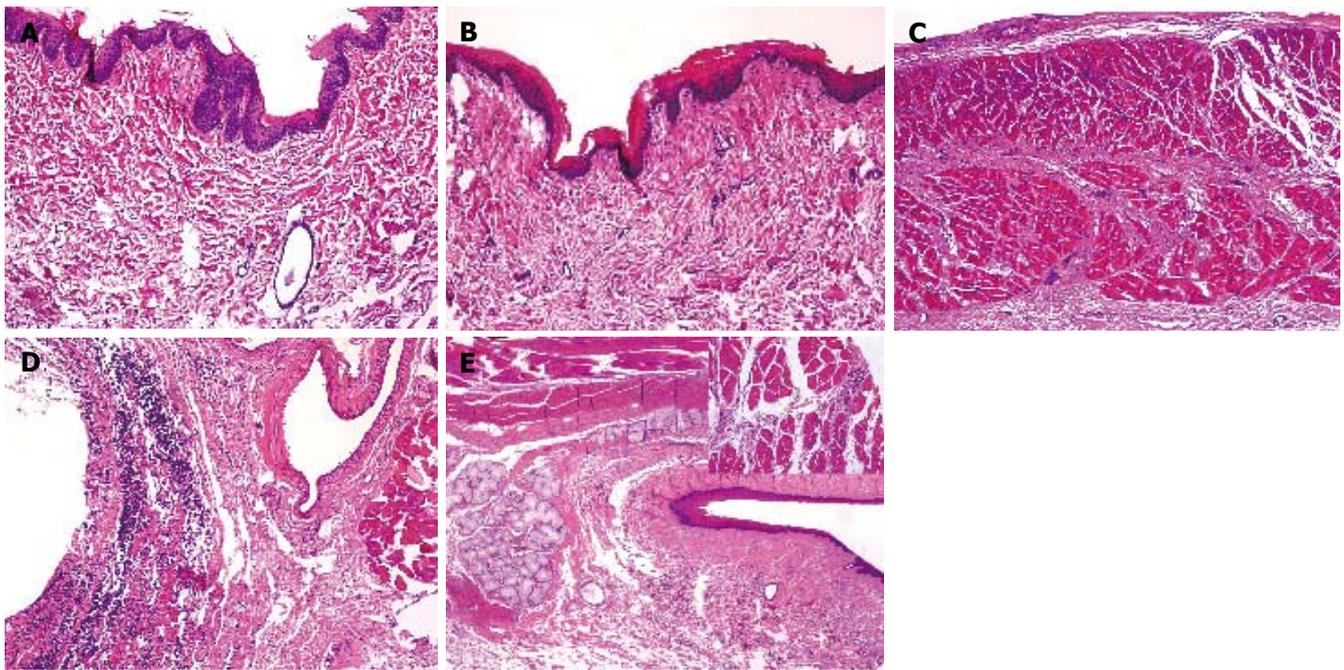


Figure 5 Photomicrographs of esophageal wall from groups 1-4 (H&E staining). **A:** control group (saline treatment, original magnification x 224), **B:** bile treatment (epithelial damage and subepithelial edema, original magnification x 112), **C:** bile-treated group (significant leukocyte infiltration in the muscle layers and adventitia, original magnification x 112), **D:** bile + 7-NI treatment (extreme leukocyte infiltration in the adventitia, original magnification x 224), and **E:** bile + PC treatment (no significant change in the esophageal wall, original magnification x 56x; the arrow in the right upper corner shows the muscle layer of the same field (original magnification x 400); the interstitial capillaries are filled with PMNs, but only few extravasated PMNs are present).

was significantly lower than that after bile treatment alone. The nNOS inhibition evoked a marked stimulation of MC degranulation over that due to bile treatment alone. In the 7-NI+bile-treated group, the degranulation of mucosal MCs was nearly complete ($M = 87.5\%$, $25p = 65.6\%$, $75p = 95.6\%$).

Biopsy samples from the saline-treated control group exhibited an average grade of injury of 5 (range of scores: 0-20) on the Lanans scale. In these sections, the luminal surface was always lined by a continuous layer of epithelial cells, while the vessels usually presented with empty lumina (Figure 5A). The 180-min bile exposure induced significant mucosal damage ($P < 0.01$), with a median value of 58 (range: 50-70). Deep lesions were observed with disruption and desquamation of the epithelial layer (Figures 5B and 5C). Extensive intraepithelial and subepithelial leukocytosis and connective tissue damage were the general characteristics. Submucosal edema, hemorrhage and vasodilatation were apparent. Semiquantitative evaluation of the samples from the 7-NI+bile-treated animals revealed a significant exacerbation of the mucosal injury ($P < 0.01$) and an injury score of 68 (range 60-90). A tendency toward more intense injury was always manifested. Severe epithelial damage and exfoliation of the surface epithelium were present, and the deeper tissue layers were more strongly involved in a generalized inflammatory reaction. In most cases, transmural inflammatory infiltrations, vasodilation and subepithelial connective tissue edema were observed (Figure 5D).

Administration of PC did not significantly influence the superficial tissue damage, but the reactive mucosal changes were different from those observed in the bile-treated group. In general, the degree of epithelial damage and the

nature of reactive epithelial changes were similar to those observed in the bile-treated group 2, but there was no evidence of severe subepithelial inflammation. Submucosal damage was often patchy, mild and severe lesions were commonly observed within the same section and hemorrhage was rarely observed. The general image of tissue damage was somewhat improved, but this group had a median grade of injury of 50 (range: 40-75), a value not significantly different ($P > 0.01$) from the corresponding value for the bile-treated group.

DISCUSSION

Regurgitation of the duodenogastric content may lead to esophageal dysfunction and tissue damage in the long run, but acute biliary reflux likewise correlates with the presence of severe esophagitis and esophageal ulceration in critically ill and mechanically ventilated patients^[16]. Appropriate treatment or prevention of this condition clearly presupposes an understanding of the pathogenesis of regurgitation-induced lesions and the nature of inflammatory complications.

The results of our *in vivo* study indicate that bile may target several potentially interconnected pathways, leading to mucosal barrier impairment. Intraluminal bile reduced ATP content of the exposed tissues, decreased cNOS activity, increased iNOS activity, evoked a parallel rise in MC degranulation and leukocyte accumulation, and induced severe structural alterations. These effects were potentiated by 7-NI, a selective inhibitor of the nNOS isoform.

NO plays a central role in the maintenance of resting esophageal mucosal blood flow, but it has been shown

that the reactive responses to luminal deoxycholate are not NO-dependent^[17, 18]. In our experiments the acute biliary challenge elicited characteristic microcirculatory changes in the esophageal mucosa, as demonstrated by intravital videomicroscopy. Both the venular diameter and RBCV increased, which shows that a substantial venodilator influence is present in the mucosa, despite cNOS inhibition. This finding is seemingly controversial. Nevertheless, it is clear that other mechanisms or mediators could compensate for the absence of cNOS-derived NO. Nonspecific inhibitors of NOS generally increase the intestinal epithelial permeability, these changes are attributed to the destabilizing effects of NO deficit on MCs^[4, 19].

It is reasonable to suggest that MC-related histamine is a likely candidate in this process. Mucosal mast MCs are a unique cellular source of both preformed and *de novo* synthesized mediators, and MC-induced reactions contribute to postischemic mucosal permeability alterations and flow response in the gastrointestinal tract^[5, 20]. Moreover, it has been shown that bile acids are able to degranulate MCs and induce histamine release from mucosal MCs both *in vitro* and *in vivo*^[3, 21]. It has further been reported that MC-derived histamine is involved in esophageal and gastric vasodilatation during acid-induced injury^[22]. This type of vasodilation could protect the mucosa against further injury and appears to be mediated by calcitonin gene-related peptides (CGRP)^[23]. Thus, it is reasonable to suggest that bile-induced venodilatory response is closely associated with MC degranulation.

The current study also showed that bile potently inhibits the constitutive, Ca²⁺-dependent isoforms of NOS, even though the activity of the inducible isoform increased over time. The latter phenomenon could be a compensatory event of cNOS inhibition, or an aftermath of inflammatory stimuli, as iNOS has been shown to be present in macrophages and MCs too. The functioning of inducible, Ca²⁺-independent iNOS is closely related to the activation of NF- κ B, and it has recently been shown that the iNOS mRNA expression increases within 30 min after an inflammatory challenge. Moreover, even physiological levels of deoxycholic acid are capable of inducing NF- κ B and NF- κ B target gene expression^[24].

Although the lowered ATP generation could be an important factor for the acute decrease in cNOS activity, the intracellular biochemical mechanisms mediating this injury are not completely understood. It has been speculated that unconjugated di- and trihydroxy bile acids cause damage by binding to and crossing cell membranes to enter cells. However, it has recently been shown that the β -chain of ATP synthase, a principal protein complex in the mitochondrial inner membrane, is also present at the cellular surface and plays a decisive role in the regulation of cell homeostasis. At the mitochondrial level, the potential toxicity of bile acids may cause cytochrome C release and Fas-dependent hepatocyte apoptosis or necrosis, and inhibit the activities of complexes I and III of the mitochondrial respiratory chain^[25]. However, because of their detergent properties, bile acids are inherently cytotoxic and their direct toxicity to isolated hepatocyte mitochondria has also been demonstrated^[26].

Some evidence additionally attests to the importance of direct effects of bile on MCs. It has been shown that lipophilic bile acids possess concentration-dependent cytotoxicity toward MCs, causing histamine release *in vitro*^[3]. Similarly, *in vivo* bile acids induce secretion in the small intestine by a mechanism involving histamine-mediated processes and MC degranulation^[27, 28]. However, Fihn *et al.*^[29] demonstrated that MC degranulation and NO release are not involved in the mechanism of deoxycholic acid-induced increase in epithelial permeability in rat small intestine^[29]. As the secretory effect of conjugated bile acids is observed only in association with an increase in mucosal permeability, a possible explanation could be that a permeability-enhancing factor is necessary to permit access of these charged large molecules to the basolateral membrane or to the subepithelium, where they can then exert their secretory effect. In our study, 7-NI treatment significantly amplified bile-induced tissue damage and caused further MC degranulation. Since 7-NI has a higher affinity for nNOS than for the eNOS isoform^[30], the residual responses to 7-NI might be mediated by a previously unblocked part of the nNOS isoform. This also suggests that bile principally inhibits eNOS, and partially sustained nNOS activity is able to counteract, or at least diminish the harmful effects of bile. This underlines the role of nNOS-derived NO in the maintenance of esophageal homeostasis.

Beside increases in blood flow, acute biliary challenge was found to be accompanied with leukocyte recruitment. In many ways, constitutively produced NO is anti-inflammatory, as it modulates the adhesive interaction between leukocytes and endothelium, and reduces MC reactivity. Thus, it could be hypothesized that decreased local ATP level, cNOS inhibition and relative lack of NO could account for the mucosal MC degranulation and the intramural blood flow increase, and these factors may play a concerted role in the process of leukocyte accumulation.

In the PC-pretreated animals, on the other hand, the bile-elicited ATP decrease, cNOS inhibition and MC degranulation were significantly attenuated. The question therefore arises as to which process may be of critical significance in the mechanism of mucosal protection after PC treatment. PC is a ubiquitous membrane-forming entity and the most prominent phospholipid species in the gastrointestinal tract. PC is present in the bile, *in vitro* and *in vivo* experiments have demonstrated that topical PC physically protects the intestinal mucosa against injurious actions of bile salts by forming less toxic mixed micelles^[31]. Nevertheless, the experimental results and clinical experience suggest that PC could function as an active substance under certain *in vivo* conditions. The therapeutic effect of dietary PC in preventing esophageal strictures due to alkali-induced esophageal burns has been demonstrated in rats^[32], and parenteral PC and lyso-PC could prolong survival in experimental sepsis models^[33, 34]. PC is taken up by phagocytic cells, and accumulates in inflamed tissues^[35]. Little is known about the transport of the molecule, but phosphatidylcholine transfer protein (PCTP) accelerates the intermembrane transfer of phospholipids in an energy-independent way. Choline, a metabolite of PC and a precursor of organic osmolyte

betaine, is actively transported by a choline carrier described in intestinal epithelial and endothelial cells of the blood-brain barrier^[36].

It is widely believed that the biological efficacy of PC depends on the fatty acid moiety^[37]. In contrast, some studies have revealed that the protective role of PC is independent of fatty acids, and it may be assumed that the active principle is choline. Phospholipase-D is activated by almost all stress factors resulting in the release of phospholipid metabolites, and several of these factors could be of importance in stress-induced defense reactions^[38]. Indeed, it has been shown that PC metabolites might relieve a potentially dangerous increase in the ratio of NADH/NAD⁺ (reductive stress), a predisposing cause of oxidative damage^[11]. This reaction sequence could explain the still incompletely understood essential role of choline in diet and its preventive efficacy in a number of experimentally induced pathologies associated with a redox imbalance. It may be assumed that the endogenous pool of these metabolites may become exhausted under exogenous provocation and that an exogenous supply might help to replenish and strengthen the endogenous protective mechanism.

In conclusion, our data suggest that nNOS-MC axis plays an important role in the mucosal defense system of the esophagus. Elucidation of the mechanisms by which bile acids induce mucosal dysfunction is complicated by the intrinsic complexity of esophageal tissue, which is made up of many different, but interacting cell types. Whether the findings in this experimental model are applicable to humans remains to be established. However, these data together with previous observations suggest a therapeutic potential for parenteral PC with a view to decreasing the harmful consequences on bile-induced tissue reactions.

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BASIC RESEARCH

Expression of vascular endothelial growth factors A and C in human pancreatic cancer

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Abstract

AIM: To study the expression of vascular endothelial growth factor A (VEGF-A) and VEGF-C and to determine whether the presence of VEGF-A and VEGF-C was associated with the clinicopathologic characteristics of pancreatic cancer.

METHODS: VEGF-A and VEGF-C mRNA transcripts were examined by Northern blot in 6 human pancreatic cancer cell lines and 8 normal pancreatic tissues and 8 pancreatic carcinoma specimens. The expression of VEGF-A and VEGF-C proteins was examined by Western blot in the tested cell lines and by immunohistochemical stain in 50 pancreatic carcinoma samples.

RESULTS: VEGF-A and VEGF-C mRNA transcripts were present in all the 6 human pancreatic cancer cell lines. Immunoblotting revealed the presence of VEGF-A and VEGF-C proteins in all the cell lines. Northern blot analysis of total RNA revealed 3.0-fold and 3.6-fold increase in VEGF-A and VEGF-C mRNA transcript in the cancer samples, respectively. Immunohistochemical analysis confirmed the expression of VEGF-A and VEGF-C in cancer cells within the tumor mass. Immunohistochemical analysis of 50 pancreatic cancer tissue samples revealed the presence of VEGF-A and VEGF-C immunoreactivity in 50% and 80% of the cancer tissue samples, respectively. The presence of VEGF-A in these cells was associated with larger tumor size and enhanced local spread ($\chi^2=6.690$, $P=0.035<0.05$) but was not associated with decreased patient survival. However, the presence of VEGF-C in the cancer cells was associated with increased lymph node metastasis ($\chi^2=5.710$, $P=0.017<0.05$),

but was not associated with decreased patient survival. There was no correlation between the expression of VEGF-A and VEGF-C in the same cancer cells.

CONCLUSION: VEGF-A and VEGF-C are commonly overexpressed in human pancreatic cancer and may contribute to tumor growth and lymph node metastasis. There is no relationship between the expression of VEGF-A and VEGF-C in pancreatic cancer.

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Key words: Pancreatic cancer; Vascular endothelial growth factor-A; Vascular endothelial growth factor-C; Survival

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INTRODUCTION

Though the mortality rate of pancreatic cancer has decreased and the survival rate of pancreatic cancer patients is improved after surgery, the 1-year survival rate after diagnosis of pancreatic cancer remains below 20% and the 5-year survival rate is only 3%^[1,2]. One reason for this poor prognosis is the propensity of pancreatic cancer to metastasize. Pancreatic carcinoma is characterized by its aggressive local invasion of adjacent structures, perineural invasion and early lymph node and liver metastasis^[3-5]. Lymph node metastasis and blood vessel invasion are the definitive factors of the prognosis after resection^[6,7].

Angiogenesis plays an important role in the development, growth and metastasis of carcinoma in mammals^[8]. It is also important to consider the possible impact of lymphangiogenesis (development of lymphatic vessels) on cancer growth and metastasis^[4]. In fact, tumor spread is dependent on both the angiogenic and lymphangiogenic systems. Many carcinomas metastasize through the lymphatic system, whereas others spread mainly hematogenously.

VEGF-A has a potent mitotic activity specific to vascular endothelial cells^[9,10], suggesting that VEGF-A

contributes to the pathologic angiogenesis associated with solid tumors. Recently VEGF-B to -E showing homology to VEGF-A have been identified^[11-13]. Among these, VEGF-C possessing approximately 30% identity with VEGF, has been shown to induce specific lymphatic endothelial proliferation and hyperplasia of the lymphatic vasculature by binding to its specific receptor flt-4^[11, 12, 14-16]. VEGF-C may also be an important factor regulating the mutual paracrine relationship between tumor cells and lymphatic endothelial cells not only in primary tumor but also in adjacent normal tissue.

We have previously reported that VEGF-C and its receptor are overexpressed in pancreatic cancer^[17]. We now report the coexpression of VEGF-A and VEGF-C in human pancreatic cancer.

MATERIALS AND METHODS

Cell culture

ASPC-1, CAPAN-1, MIA-PaCa-2 and PANC-1 human pancreatic cancer cells were obtained from the American Type Culture (Rockville, MD, USA). COLO-357 and T3M4 human pancreatic cancer cells were a gift from R. S. Metzger at Duke University. Cells were grown in monolayer culture containing humidified 50ml/L CO₂ and 95% air at 37°C. ASPC-1, CAPAN-1 and T3M4 cells were grown in RPMI-1640 medium. COLO-357, MIA-PaCa-2 and PANC-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM). Media contained 10% fetal bovine serum, 100 U/mL penicillin and 100 mg/mL streptomycin.

Tissues for Northern blot analysis

Normal pancreatic tissue and pancreatic cancer tissue samples were frozen in liquid nitrogen and stored at -80°C for RNA extraction.

Tissues for immunohistochemistry

Pancreatic cancer tissue samples were obtained from 50 patients (35 men and 15 women; mean age, 64.3 ± 10.8 years; range, 48-81 years) undergoing surgery for pancreatic cancer. The tumors were classified as previously described^[18]. Histologically, there were 15 well, 31 moderately and 4 poorly differentiated adenocarcinomas. There were 0 stage I, 3 stage II, 9 stage III and 38 stage IV tumors. Tissue samples were fixed in 10% paraformaldehyde solution for 18-24 h and embedded in paraffin.

Cloning and labeling of VEGF-A and VEGF-C probes

Reverse-transcription polymerase chain reaction (RT-PCR) was carried out using RT-PCR kit according to the manufacturer's instructions. Briefly, total RNA was extracted from human placenta using the acid guanidinium thiocyanate method. The prepared RNA (1 µg) was mixed with reverse transcription reagents in a total volume of 20 µL, incubated for 30 min at 42 °C into first-strand cDNA. One µL cDNA was used for PCR amplification. The upstream primer for VEGF-A was 5'-CTC ACC TGC TTC TGA GTT GC-3', and the downstream primer was 5'-TTC TCT GCC TCC ACA ATG G-3' with an expected size of 319 bp. The upstream primer for VEGF-C was 5'-CGGGATCCACGGCTTATGCAAGCAAA-3', and the

downstream primer was 5'-CGGAATTCAACACAGTT TTCCATAATAGA-3 with an expected size of 1167 bp. Amplification conditions were denaturation at 94 °C for 5 min, followed by 32 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min. The PCR products were electrophoresed on 1.5% agarose gels and the target bands containing the specific cDNA were cut down and purified using an ultraclean DNA purification kit (Mo Bio Laboratories, Solana Beach, CA, USA.) according to the manufacturer's instructions. The accuracy of the RT-PCR products was confirmed by sequencing of cloned cDNA. The prepared cDNA probes were labeled by PCR-DIG labeling mix according to the manufacturer's instructions.

Northern blot analysis

Total RNA was extracted from human tissue samples and human pancreatic cancer cell lines using the acid guanidinium thiocyanate method. RNA was fractionated on 0.8% agarose/2.2% formaldehyde gels, electrotransferred onto nylon membranes and cross-linked by ultraviolet irradiation^[19]. The blots were prehybridized in hybridization buffer for 1 h at 42 °C, hybridized with the indicated DIG-labeled cDNA probes overnight at 42°C, and washed twice for 5 min with 2×SSC/SDS (0.1%) at room temperature, twice for 15 min with 0.1×SSC/SDS (0.1%) at 68°C. DIG-labeled nucleic acids were detected with CSPD according to the manufacturer's instructions at room temperature. Briefly, after rinsed in washing buffer, the membrane was incubated for 30 min in blocking solution and anti-DIG-AP conjugate, respectively. Then the membrane was washed twice in washing buffer for 15 min and in detection buffer for 5 min and incubated for 5 min in CSPD solution. The damp membrane was sealed in a hybridization bag, incubated for 15 min at 37 °C and exposed to radiographic film for 15 min at room temperature. Equivalent loading of RNA in each lane was confirmed by hybridizing the total RNA with a β-actin cDNA. All cDNAs were labeled with DIG as above. Densitometric analysis of the signals were performed with a gel imaging system.

Immunoblotting

Human pancreatic cancer cells were solubilized in lysis buffer containing 50 mmol Tris, 150 mmol NaCl, 1 mmol ethyleneglycoltetraacetic acid, 1% NP40, 1% sodium deoxycholate, 1 mmol sodium banadate, 50 mmol sodium flupride, 2 mmol EDTA (pH 8.0), 100 µg/mL benzamidine, 50 µg/mL aprotinin, 10 µg/mL leupeptin, 10 µg/mL pepstatin A, and 1 mmol phenylmethylsulfonyl fluoride. Proteins were subjected to sodium dodecyl sulfatepolyacrylamide gel electrophoresis and transferred to Immobilon P membrane. Membranes were incubated for 90 min with highly specific anti-VEGF-A or anti-VEGF-C polyclonal antibody, then washed and incubated with a horseradish peroxidase-coupled secondary goat anti-rabbit antibody for 60 min. After washing, antibodies were visualized by enhanced chemiluminescence. Equivalent loading of protein in each lane was confirmed by hybridizing the total protein with β-actin.

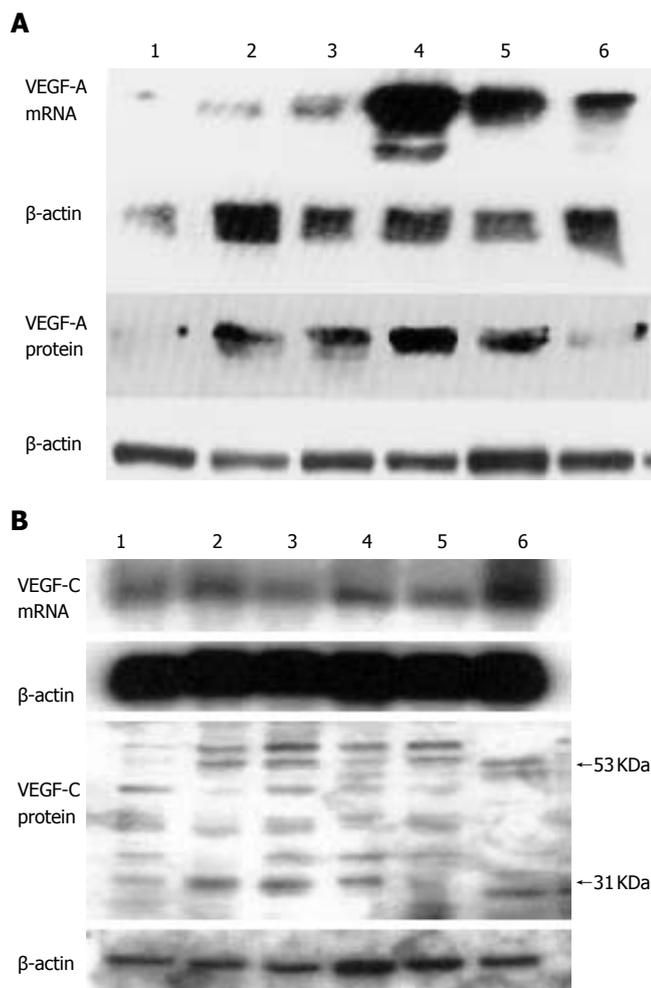


Figure 1 Expression of VEGF-A (A) and VEGF-C (B) in six human pancreatic cancer cell lines. Lane 1: COLO-357; lane 2: MIA-PaCa-2; lane 3: PANC-1; lane 4: T3M4; lane 5: ASPC-1; lane 6: CAPAN-1.

Immunohistochemistry

The same anti-VEGF-A and anti-VEGF-C polyclonal antibodies were used for immunohistochemical analysis. Paraffin-embedded sections (4 μ m) of pancreatic cancer and noncancerous pancreatic tissues were subjected to immunostaining using the streptavidin-peroxidase technique. After deparaffinization, antigen retrieval was performed by immersing the sections in 10 mmol citrate buffer (pH 6.0) and heated twice in a microwave oven (95 $^{\circ}$ C) for 5 min each time. Endogenous peroxidase activity was blocked by incubation with 1% hydrogen peroxide in distilled water for 10 min. Tissue sections were incubated with normal donkey serum for 20 min at room temperature and then incubated with polyclonal anti-VEGF-A antibody (2 μ g/mL) or anti-VEGF-C antibody (2 μ g/mL) in normal donkey serum for 2 h at room temperature. Bound antibodies were detected with biotinylated goat anti-rabbit IgG secondary antibody and streptavidin-peroxidase complex, using diaminobenzidine tetrahydrochloride as the substrate. The sections were counterstained with Mayer's hematoxylin and incubated with nonimmunized rabbit IgG or without primary antibodies, which did not yield positive immunoreactivity.

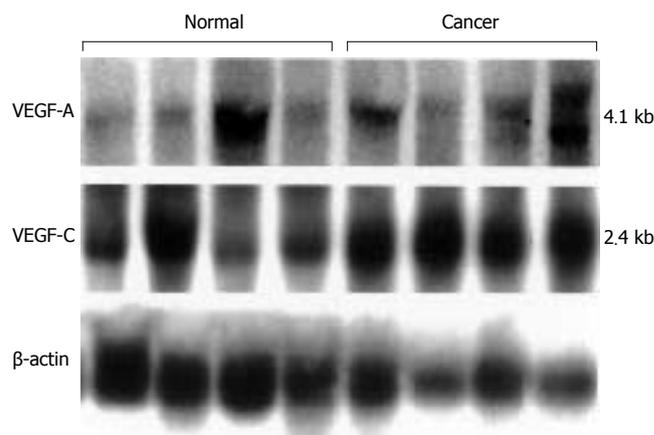


Figure 2 Northern blot analysis of VEGF-A and VEGF-C expression in human pancreatic tissues.

Scoring was carried out by two independent observers blinded to the patient's status. Positive staining was defined as the presence of VEGF-A and VEGF-C immunoreactivity in at least 10% of the cancer cells.

Statistical analysis

Differences in distribution of VEGF-A or VEGF-C were determined using the χ^2 test. Kaplan-Meier survival analysis was used to estimate survival time and log-rank test was used to compare differences in survival time between VEGF-A positive and negative groups or between VEGF-C positive and negative groups^[20]. $P < 0.05$ was considered statistically significant.

RESULTS

Expression of VEGF-A and VEGF-C in human pancreatic cancer cell lines

Northern blot analysis of total RNA isolated from pancreatic cancer cell lines indicated that the 6 cell lines expressed the 4.1 kb VEGF-A transcript and 2.4 kb VEGF-C transcript (Figures 1A and 1B). Immunoblotting with a highly specific anti-VEGF-A or anti-VEGF-C antibody revealed an approximately M_r 43000 band (occasionally also a M_r 41000 band) of VEGF-A protein or M_r 55000 band (occasionally proteolytic forms of M_r 41000 molecules) of VEGF-C protein in all the cell lines (Figures 1 A and 1B). Both M_r 43000 band and M_r 41000 band corresponded to the VEGF165 isoform homodimer.

Expression of VEGF-A and VEGF-C mRNA in human pancreas

Northern blot analysis of total RNA isolated from human pancreatic tissue revealed the 4.1 kb VEGF-A transcript and the 2.4 kb VEGF-C transcript in all normal pancreatic tissue samples and cancer tissue samples (Figure 2). Densitometric analysis of the autoradiograms indicated that the levels of 4.1 kb VEGF-A transcript and 2.4 kb VEGF-C transcript were approximately 3.0-fold and 3.6-fold higher in pancreatic cancer tissues in comparison with normal pancreatic tissues.

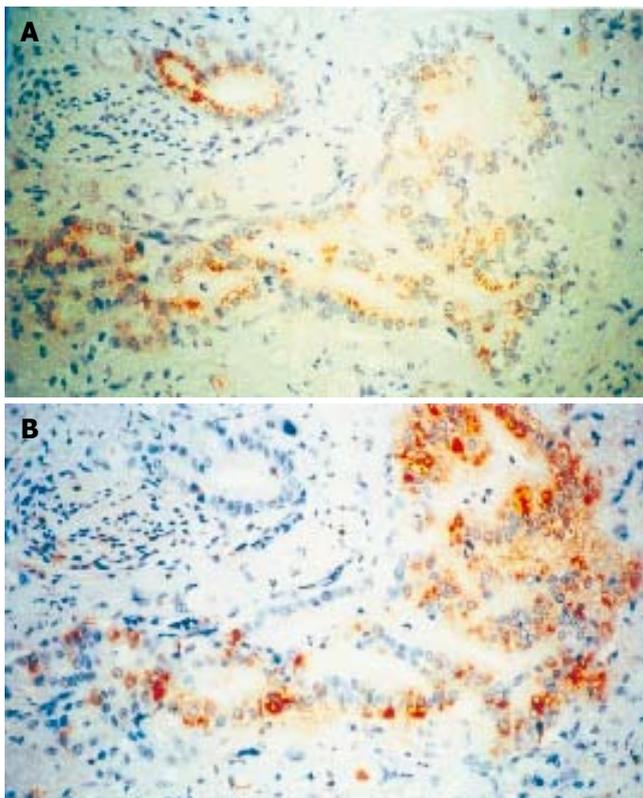


Figure 3 Immunohistochemical analysis of VEGF-A (A) and VEGF-C (B) expression in human pancreatic cancer tissues. In the pancreatic cancers, moderate to strong VEGF-A and VEGF-C immunoreactivity was present in the cytoplasm of cancer cells, $\times 400$.

Expression of VEGF-A and VEGF-C protein in human pancreatic tissue

The same anti-VEGF-A or anti-VEGF-C antibody was used to localize VEGF-A or VEGF-C immunohistochemically. In the normal pancreatic tissue, moderate VEGF-A and VEGF-C immunoreactivity was present in the cytoplasm of endocrine islet cells, a weak immunoreactivity in some ductal cells within the small ductules and in a few acinar cells (data not shown). In the pancreatic cancer tissue, moderate to strong VEGF-A and VEGF-C immunoreactivity was present in the cytoplasm of many cancer cells (Figures 3A and 3B). Among the 50 pancreatic cancer cases, 25 (50%) were positive for VEGF-A and 40 (80%) for VEGF-C immunoreactivity in the cytoplasm of cancer cells.

Correlation between VEGF-A and VEGF-C expression and patient survival

χ^2 analysis indicated that the presence of VEGF-A in cancer cells was associated with a statistically significant increase in tumor size and local extension (T category). However, there was no correlation between the presence of VEGF-A in cancer cells and lymph node metastasis or distant metastasis or tumor stage or histological grade of cancers (Table 1). The presence of VEGF-C in cancer cells was associated with a statistically significant increase in the presence of lymph node metastasis (N category), but there was no correlation between the presence of VEGF-C in cancer cells and other factors (Table 1). The

Table 1 VEGF-A and VEGF-C expression in relation to the clinicopathologic characteristics of 50 patients with pancreatic cancer

| | VEGF-A | | | VEGF-C | | |
|-----------------------|-------------|------------|-------|-------------|------------|-------|
| | + | - | P | + | - | P |
| Age (yr) | 64 \pm 10 | 62 \pm 9 | | 65 \pm 10 | 62 \pm 8 | |
| Gender | | | | | | |
| Male | 18 | 17 | | 28 | 7 | |
| Female | 7 | 8 | | 12 | 3 | |
| TNM | | | | | | |
| T1 | 1 | 8 | 0.035 | 8 | 1 | 0.701 |
| T2 | 9 | 7 | | 13 | 3 | |
| T3 | 15 | 10 | | 19 | 6 | |
| N0 | 6 | 5 | 0.733 | 6 | 5 | 0.017 |
| N1 | 19 | 20 | | 34 | 5 | |
| M0 | 20 | 23 | 0.221 | 35 | 8 | 0.541 |
| M1 | 5 | 2 | | 5 | 2 | |
| Stage | | | | | | |
| I | 0 | 0 | 0.801 | 0 | 0 | 0.416 |
| II | 1 | 2 | | 2 | 1 | |
| III | 5 | 4 | | 6 | 3 | |
| IV | 19 | 19 | | 32 | 6 | |
| Differentiated degree | | | | | | |
| well | 10 | 5 | 0.290 | 14 | 1 | 0.304 |
| moderate | 13 | 18 | | 23 | 8 | |
| poor | 2 | 2 | | 3 | 1 | |

TNM: tumor node metastasis.

Table 2 Correlation between VEGF-A and VEGF-C expression in 50 patients with pancreatic cancer

| | VEGF(-A) | | P |
|--------|----------|----|-------|
| | + | - | |
| VEGF-C | + 21 | 19 | 0.480 |
| | - 4 | 6 | |

mean survival time of the VEGF-A negative and positive groups was 20.80 \pm 15.00 months and 16.04 \pm 9.00 months respectively. Thus, the survival time of patients with VEGF-A positive tumors was shorter. However, Kaplan-Meier analysis and log-rank test indicated that there was no significant difference in survival between these two groups (Figure 4A). The mean survival time of VEGF-C negative and positive groups was 30.7 \pm 14.00 months and 15.4 \pm 10.00 months respectively. The survival time of patients with VEGF-C positive tumors was shorter. But Kaplan-Meier analysis and log-rank test indicated that there was no significant difference in survival between these two groups (Figure 4B).

Correlation between VEGF-A and VEGF-C expression in pancreatic cancer

There was no correlation between VEGF-A and VEGF-C expression in pancreatic cancer (Table 2).

DISCUSSION

Recently, VEGF-B to -E showing homology to VEGF-A have been identified^[11-13]. Among these, VEGF-C has

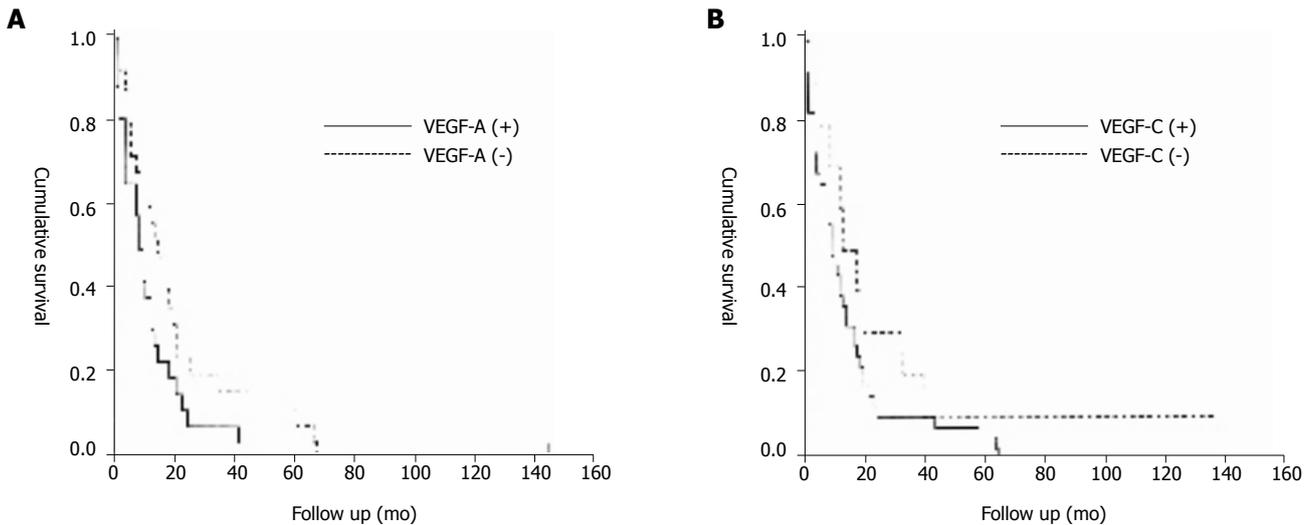


Figure 4 Survival curve. **A:** Cumulative survival (Kaplan-Meier) plot of the postoperative survival period of patients whose pancreatic tumors exhibited cytoplasmic VEGF-A immunostaining (solid line) versus those whose tumors were negative for VEGF-A (broken line), ($P=0.184$); **B:** Cumulative survival (Kaplan-Meier) plot of the postoperative survival period of patients whose pancreatic tumors exhibited cytoplasmic VEGF-C immunostaining (solid line) versus those whose tumors were negative for VEGF-C (broken line), ($P=0.159$).

a high degree (approximately 30%) of homology to VEGF(-A)^[11, 12, 14-16]. VEGF-A plays an important role in tumor growth and metastasis^[21-23]. VEGF-C is a ligand for VEGF-A receptor 3 (flt-4) expressed mainly in endothelia of lymphatic vessels and induces specific lymphatic endothelial proliferation and hyperplasia of the lymphatic vasculature^[11, 24].

Expression of VEGF-A and VEGF-C was detected in normal and cancer tissues in the present study. Overexpression of VEGF-A has been reported in mammary, colorectal, liver, gastric and pancreatic malignancies^[21, 22, 25-27]. Expression of VEGF-A and its receptor KDR correlates with vascularity, proliferation and metastasis of colonic cancer^[22] and with vessel count and tumor stage of gastric cancer^[26] as well as with growth of pancreatic cancer. Recent studies demonstrated that expression of VEGF-C in various human tumors, including lymphoma, melanoma, fibrosarcoma, squamous cell carcinoma, breast cancer, gastric cancer, colorectal cancer, pancreatic cancer and hepatocellular carcinoma^[17, 28-34]. VEGF-C is expressed only in lymph node-positive breast cancer^[29]. Gastric cancer patients with a high expression of VEGF-C protein have a much poorer prognosis than those with a low VEGF-C expression^[14]. VEGF-C promotes lymph node metastasis of rectal cancer^[30]. Expression of VEGF-C and its receptor flt-4 correlates with lymphangiogenic process and metastasis of pancreatic cancer^[17].

The present study showed that VEGF-A and VEGF-C expression was abnormal in pancreatic cancer *in vivo*. Northern blot analysis revealed a single 4.1 kb VEGF-A mRNA transcript and a single 2.4 kb VEGF-C mRNA transcript in all normal and pancreatic cancer tissue specimens, as well as 3.0-fold and 3.6-fold increase of VEGF-A and VEGF-C mRNA transcripts in pancreatic cancer tissue specimens, indicating that both VEGF-A and VEGF-C are overexpressed in pancreatic cancer. The distribution of VEGF-A and VEGF-C in normal and

cancerous tissue specimens was different. In the normal pancreas, VEGF-A and VEGF-C were relatively abundant in endocrine islets, less frequently present in ductal cells and only occasionally present in acinar cells. In contrast, VEGF-A and VEGF-C were abundant in ductal-like cancer cells of pancreas. VEGF-A is a specific mitogen toward endothelial cells and VEGF-C is a specific mitogen to lymphatic endothelial cells, suggesting that VEGF-A has a potential to act in a paracrine manner on endothelial cells within the pancreatic tumor mass, leading to enhanced angiogenesis and tumor growth, while VEGF-C has a potential to act in a paracrine manner on lymphatic endothelial cells within the pancreatic tumor mass, leading to enhanced invasion to lymphatic vessels and lymph node metastasis. Cancer with active lymphangiogenesis could be predisposed to metastatic spread via the lymphatic system, resulting in a poor survival rate. It appears that tumor cells need to penetrate the lymphatic endothelium twice to translocate into the interstitium of a lymph node. Because lymphatic vessels have no tight junctions or continuous basal laminae, their penetration may only require tumor cell adhesion to the endothelium and transmigration through intracellular gaps between lymphatic endothelial cells. VEGF-A can increase microvascular permeability by enhancing the transmigration activity of vesicular-vacuolar organelles^[32-34]. VEGF-C and flt-4 may be components of a paracrine signaling network between cancer cells and the endothelium and may be involved in modifying the permeabilities of both blood and lymphatic vessels and metastasis^[35].

Univariate analysis of the immunohistochemical data demonstrated that the presence of VEGF-A and VEGF-C in pancreatic cancer cells was associated with tumor size and lymph node metastasis respectively, indicating that VEGF-A and VEGF-C contribute to pancreatic tumor growth and lymphatic metastasis *in vivo*. Though the survival time was shorter in patients with VEGF-A and VEGF-C positive tumors, this correlation was not statisti-

cally significant. Niedergethmann *et al.*^[36] and Kuwahara *et al.*^[37] reported that the survival time of patients with pancreatic ductal carcinoma showing high expression of VEGF-A is significantly shorter than that of those with low or no expression of VEGF-A. Kurahara *et al.*^[38] reported that VEGF-C and VEGF-D expression in tumor cells is significantly associated with the 5-year survival rate of patients with pancreatic head cancer. In our study, VEGF-A or VEGF-C did not emerge as an independent prognostic parameter. Moreover, our previous study showed that the survival time of patients with VEGF-C positive tumors is shorter^[17]. Because the majority of patients in the present study had no detectable metastatic disease at the time of surgery, whether VEGF-A or VEGF-C expression correlates with enhanced propensity of pancreatic cancer to metastasize remains unknown. Further studies are needed.

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Role of N-acetylcysteine in rifampicin-induced hepatic injury of young rats

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Abstract

AIM: To study the role of N-acetylcysteine (NAC) as a protective agent in rifampicin (RMP)-induced oxidative hepatic injury of young rats.

METHODS: Hepatic injury was produced by giving 50mg/kg body weight/day of RMP for 3 wk. A dose of NAC (100mg/kg body weight/day) was given in combination with RMP intraperitoneally. Analysis of lipid peroxidation, thiol levels, cytochrome P₄₅₀, superoxide dismutase (SOD), catalase, glutathione peroxidase, reductase and transferase were estimated in liver along with the body weight, liver weight and histological observations.

RESULTS: RMP exposure resulted in no change in body and liver weight while antioxidative enzymes were altered but the non protein thiol (GSH) status was well preserved. Cytochrome P₄₅₀ system and peroxidation of lipids were induced by RMP exposure. Partial protection was observed with NAC against RMP-induced changes in liver, which was evidenced from the prevention of increase in lipid peroxidation and the reduction in SOD and catalase enzyme levels.

CONCLUSION: NAC protects young rats against RMP-induced oxidative hepatic injury.

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Key words: Rifampicin; N-acetylcysteine; Hepatoprotection.

Rana SV, Attri S, Vaiphei K, Pal R, Attri A, Singh K. Role of N-acetylcysteine in rifampicin-induced hepatic injury of

INTRODUCTION

Tuberculosis is one of the major health problems in developing countries like India. Most adult deaths in India are due to vascular diseases or pulmonary tuberculosis^[1]. Steel *et al*^[2] reported that clinical hepatitis occurs in 1.1% of adults receiving RMP containing regimens. Occasional cases of RMP-associated hepatitis have been reported in patients not receiving isoniazid (INH) treatment^[3]. The actual incidence of RMP-induced hepatitis remains unclear because RMP is always used in combination with other antitubercular drugs.

The mechanism of RMP-induced liver injury is not yet fully understood. Gangadharan^[4] showed that RMP causes a direct toxic injury to the hepatocytes. This has been confirmed in experimental rats by Sodhi *et al*^[5]. Hepatotoxicity still remains a definite yet unpredictable risk during treatment of tuberculosis patients, suggesting that diminution of protective mechanisms and enhancement of destructive mechanism might be important in RMP-induced hepatotoxicity.

It is well established that by augmenting cellular antioxidative defense system especially non protein thiols, i.e. glutathione (GSH), cells can be protected against oxidative injuries produced by various drugs^[6]. Among the possible antioxidant chemicals, NAC known to be non-toxic has been used in the treatment of various disorders^[7, 8]. The present study was designed to see whether NAC could protect against RMP-induced oxidative hepatic injury in an experimental model.

MATERIALS AND METHODS

Animals

Young male Wistar rats weighing about 150-200 g were selected in the present study. Animals were fed with standard rat pellet diet (Aashirwad Industries, Chandigarh) and water *ad libitum*.

Drugs

NAC was obtained from Sigma Chemical Company, USA. RMP and NAC were administered intraperitoneally (i.p) to animals. RMP solution was prepared by dissolving the powder in sterile distilled water and then the pH was

adjusted to 3.0 with 0.1 N HCl to have a clear solution^[9]. Similarly, NAC was dissolved in sterile distilled water. The volume of sterile water injected either alone or containing drugs, was kept constant (4.0mL/kg body weight/day) in all the treatments.

A dose of NAC (100 mg/kg body weight/day) was selected when the animals treated with this dose did not exhibit any histopathological liver injury at the end of the treatment schedule.

Study protocol

Forty animals were divided into four groups (10 each group) and injected with RMP and / or NAC (dissolved in sterile water) for a period of three weeks.

Animals in control group were injected with sterile water alone. Animals in RMP group were injected with 50mg/kg body weight/day of RMP alone after dissolved in sterile distilled water. Animals in RMP+NAC group were injected with RMP (50mg/kg body weight/day) and NAC (100mg/kg body weight/day). Animals in NAC group were treated with NAC at a dose of 100mg / kg body weight / day.

Animals were fasted for 12 h before sacrificed by cervical dislocation under light anesthesia and weighed on d 0 and 10 and at the time of sacrifice. Their liver tissues were excised and cooled in an ice-bath after weighed. Liver tissues were perfused with ice-cold saline. Homogenate and post mitochondrial supernatant were prepared as previously described^[5]. Crude homogenate (20%) was prepared in 100mmol/L phosphate buffer and used to estimate thiols (total and protein-bound) and lipid peroxidation. The homogenate was then centrifuged at 10000r/min for 20 min at 4°C. The post mitochondrial supernatant (PMS) was separated from the pellet by decantation and pellet was discarded. All enzyme assays were carried out using PMS fraction.

Estimation of thiols was carried out according to the method of Sedlak and Lindsay^[10]. Lipid peroxidation was measured by the method of Ohkawa *et al*^[11]. Cytochrome P₄₅₀ was assayed using the method of Omura and Sato^[12]. The method of Kono^[13] was adopted to estimate superoxide dismutase. Activity of catalase was measured by the method of Luck^[14]. Glutathione peroxidase, glutathione reductase and glutathione transferase were estimated using the methods of Flohe and Gungler^[15], Carlberg *et al*^[16] and Habig *et al*^[17], respectively. Proteins were estimated using bovine serum albumin as standard by the method of Lowry *et al*^[18].

Histopathological investigation

Small pieces of liver tissue from sacrificed animals were preserved in 10% formal saline. Light microscopy was performed after slides were routinely stained with haematoxylin and eosin (H&E).

Statistical analysis

Data were analysed by analysis of variance (ANOVA) followed by multiple comparison using Dunnett's procedure to compare all groups against control and Student-Newman-Keul's procedure to compare the pair wise of all groups.

An approval from Postgraduate Institute of Medical

Education and Research Ethical Committee was obtained before the animal experimentation.

RESULTS

Analysis of variance showed that inter-group variation in body weight and the effect of treatment on body weight were not significant. Relative liver weight (g/100 g body weight) of experimental animals was not affected by any of the treatments. Animals treated with RMP had comparable liver weight (4.75 ± 0.75g) to animals treated with RMP+NAC (4.85 ± 0.67g).

Mortality in RMP treatment group was decreased from 30% (3/10) to 10% (1/10) when NAC was co-administered with RMP.

Essentially normal morphology was observed in all 10 control animals (100%) (Figure 1). Hepatotoxicity was produced by i.p. injection of RMP at a dose of 50mg / kg body weight / day over a period of three weeks as evidenced by the presence of changes such as hepatocytic necrosis and portal triaditis. Nine out of 10 (90%) animals treated with RMP showed histological lesions, 7 out of 10 (70%) animals had moderate degree of portal triaditis (Figure 2A) and 2 out of 10 (20%) had spotty necrosis with portal triaditis (Figure 2B). Only 1 out of 10 (10%) animals treated with RMP showed normal morphology.

Animals co-exposed to RMP+NAC exhibited lower degrees of histological lesions such as mild to moderate portal triaditis and spotty necrosis. Control animals treated with NAC alone had no change in liver histology.

Hepatic thiols were significantly increased ($P < 0.05$) after treatment with RMP, while NAC had no effect on total and bound thiols when animals were treated with RMP+NAC. Multiple comparisons using Newman-Keul's procedure did not reveal any significant change in treated groups with respect to non protein thiols (GSH) (Table 1).

Lipid peroxidation was significantly ($P < 0.001$) enhanced in animals exposed to RMP compared to the controls. Simultaneous administration of NAC and RMP prevented the enhancement of lipid peroxidation compared to control animals ($P < 0.05$). But the values of lipid peroxidation in RMP+NAC-treated animals were higher ($P < 0.05$) than those in the controls (Table 2). There was no significant difference in LPO values between NAC-treated group and controls.

Superoxide dismutase (SOD) was decreased significantly ($P < 0.001$) in RMP-treated animals compared to controls. Supplementation of NAC to RMP treated animals significantly prevented the reduction of superoxide dismutase while no effect of NAC was observed in control group (Table 2).

Catalase activity was significantly decreased in RMP-treated animals as compared to controls ($P < 0.001$). Supplementation of NAC to RMP-treated animals prevented the lowering of catalase levels. However, animals treated with NAC in combination with RMP had significantly lower ($P < 0.001$) enzyme levels compared to the controls while NAC alone had no effect on catalase activity in control group (Table 2).

Levels of glutathione-S-transferase were significantly higher ($P < 0.001$) in RMP-treated group than in controls.

Table 1 Effect of RMP treatment (50 mg/kg/day for 3 wk) and NAC (100 mg/kg/day for 3 wk) on total, protein-bound and non-protein thiols in rats ($\mu\text{mole/g}$ liver) (mean \pm SD)

| Group | Total thiols | Protein bound | Non protein thiols (GSH) |
|-----------|-------------------------------|-------------------------------|--------------------------|
| Control | 13.42 \pm 0.52 | 10.09 \pm 0.45 | 3.23 \pm 0.42 |
| RMP | 16.77 \pm 0.61 ^a | 13.49 \pm 0.42 ^a | 2.98 \pm 0.61 |
| RMP + NAC | 17.17 \pm 0.78 ^b | 13.92 \pm 0.51 ^b | 3.37 \pm 0.72 |
| NAC | 13.44 \pm 0.50 | 9.55 \pm 0.25 | 3.42 \pm 0.43 |

^a $P < 0.05$ vs control, ^b $P < 0.001$ vs control.**Table 3** Effect of RMP treatment (50 mg/kg/day for 3 wk) and NAC (100 mg/kg/day for 3 wk) on glutathione peroxidase, glutathione reductase and cytochrome P₄₅₀ (nmol/min/mg protein) (mean \pm SD)

| Group | Glutathione peroxidase | Glutathione reductase | Cytochrome P ₄₅₀ |
|-------------|----------------------------------|-----------------------|--------------------------------|
| Control (C) | 458.37 \pm 45.19 | 25.37 \pm 3.82 | 0.431 \pm 0.050 |
| RMP | 287.18 \pm 39.82W ^b | 27.01 \pm 2.75 | 0.540 \pm 0.039 ^b |
| RMP + NAC | 385.99 \pm 50.47 ^a | 27.47 \pm 3.27 | 0.509 \pm 0.042 ^a |
| NAC | 474.07 \pm 37.92 | 26.93 \pm 4.01 | 0.412 \pm 0.047 |

^a $P < 0.05$ vs control, ^b $P < 0.001$ vs control.

Supplementation of NAC to RMP-treated animals prevented the increase of GST activity. GST level in NAC-treated animals was comparable to that in the controls (Table 2).

Glutathione peroxidase activity was significantly ($P < 0.001$) decreased after RMP treatment. NAC in combination with RMP could prevent the decrease of glutathione peroxidase activity but was still significantly lower than that in the controls ($P < 0.05$). NAC had no effect on this enzyme in control group (Table 3).

Glutathione reductase did not significantly increase in the RMP-treated animals compared to the controls. Supplementation of NAC to RMP-treated and control groups had comparable enzyme activities to non RMP-exposed animals (Table 3).

Cytochrome P₄₅₀ was induced in RMP-treated animals ($P < 0.001$). Supplementation of NAC to RMP-treated and control animals had no effect on cytochrome P₄₅₀ in the present study. The values in RMP+NAC group were comparable to those in RMP-treated animals (Table 3).

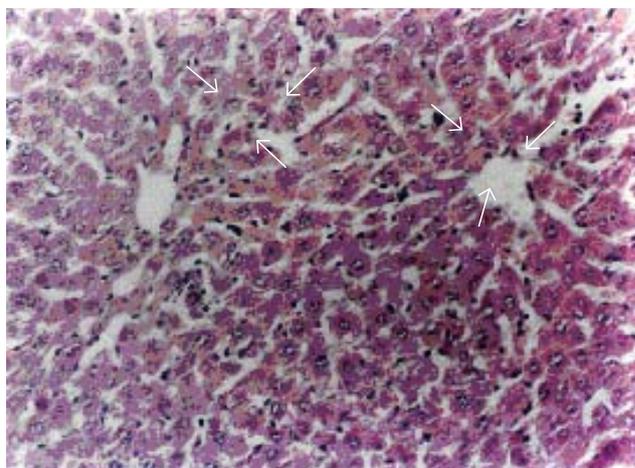
DISCUSSION

Rat model was used to study the hepatotoxic effect of RMP and hepatoprotective effect of NAC. Rats have been successfully used to establish RMP-induced hepatotoxicity models^[9,19]. Our earlier studies have shown that oxidative stress is the mechanism of RMP-induced hepatotoxicity in experimental rats^[5].

In the present study, hepatotoxicity was produced by RMP at a dose of 50 mg/kg body weight/day for three weeks. The dose is very high compared to that in the

Table 2 Effect of RMP treatment (50 mg/kg/day for 3 wk) and NAC (100 mg/kg/day for 3 wk) on lipid peroxidation, catalase, superoxide dismutase, glutathione-S-transferase (nmol/min/mg protein) in rats (mean \pm SD)

| Group | Lipid peroxidation (nmol MDA/g tissue /10min) | Catalase ($\mu\text{mol/min/mg}$ protein) | superoxide dismutase (IU) | Glutathione-S-transferase using EA |
|-----------|---|--|--------------------------------|------------------------------------|
| Control | 120.47 \pm 9.3 | 310.32 \pm 31.2 | 17.92 \pm 2.25 | 13.94 \pm 2.27 |
| RMP | 163.52 \pm 22.6 ^b | 204.92 \pm 28.6 ^b | 11.71 \pm 1.39 ^b | 18.58 \pm 1.52W ^b |
| RMP + NAC | 138.71 \pm 13.2W ^a | 269.48 \pm 19.5W ^a | 14.38 \pm 2.47W ^a | 15.92 \pm 1.38 ^a |
| NAC | 103.59 \pm 12.9 | 317.51 \pm 38.7 ^a | 16.47 \pm 2.80 | 14.59 \pm 2.18 |

^a $P < 0.05$ vs control, ^b $P < 0.001$ vs control.**Figure 1** Normal morphology of rat liver (H&E, X 55).

treatment of tuberculosis in human subjects, because higher doses are required for animals like rats as they metabolize the drugs at a faster rate^[20].

In the present study, the drugs used had no effect on body weight and relative liver weight of the animals. This is consistent with what has been reported earlier^[21].

Cytochrome P₄₅₀ mediates generation of reactive metabolites of drugs and their covalent binding to hepatic macromolecules is the most accepted mechanism of RMP-induced hepatic injury^[22]. RMP is a potent inducer of cytochrome P₄₅₀ and enhances the covalent binding of reactive metabolites of acetyl hydrazine to the macromolecules of hepatocytes^[21].

In the present study, free radicals formed either by the reaction of drug's radicals with oxygen or by the interaction of superoxide radicals with hydrogen peroxide seemed to initiate lipid peroxidation in RMP-treated rats, suggesting that increased lipid peroxidation might be associated with cellular damage. NAC combined with RMP prevented significantly the peroxidation of lipids in animals exposed to RMP either directly or through non-protein thiols (GSH) by scavenging the radicals. The scavenging ability of NAC has also been reported during cancer chemotherapy^[23, 24]. From these studies and results of the present study, it can be concluded that the protective effect of NAC may be due to the radicals scavenged by NAC.

SOD, catalase and glutathione peroxidase constitute a

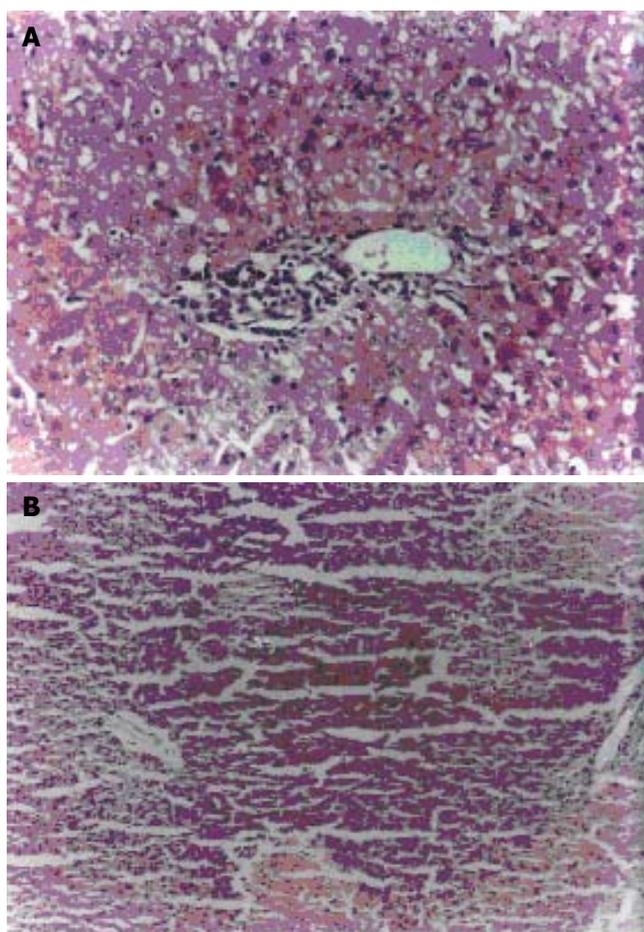


Figure 2 Portal triaditis (A) and spotty necrosis (B) in rats treated with RMP (H&E, X55)

mutually supportive team of antioxidative enzymes which provide a defense against reactive oxygen species. In the present study, SOD decreased significantly in RMP-exposed animals. The activities of H_2O_2 scavengers, catalase and glutathione peroxidase, decreased significantly after RMP treatment. Similar results have been reported by Sodhi *et al*^[5]. The decline in these enzymes in the present study could be explained by the fact that excess superoxide radicals may inactivate H_2O_2 scavengers, thus resulting in inactivation of SOD^[25].

In the present study, co-administration of NAC and RMP partially prevented decrease in catalase and glutathione peroxidase activities, which might be due to incomplete scavenging of radicals by NAC, resulting in partial protection of these enzymes. As NAC reacts with scavenging radicals slowly, residual superoxide radicals might interact with H_2O_2 resulting in the formation of OH radicals. This is supported by the fact that lower increase in lipid peroxidation was observed in NAC+RMP co-exposed animals as compared to RMP-treated animals. Jaya *et al*^[26] have also reported that anti-peroxidative enzymes can be partially restored with NAC in rats exposed to alcohol and paracetamol.

It was reported that thiols increase significantly after RMP treatment, which might be due to the increased protein content^[28].

GSH constitutes approximately 90% of total hepatic

thiols, which could explain the maintained GSH levels in RMP-exposed animals in the present study. Supplementation of NAC also failed to increase the threshold levels of GSH. These results are inconsistent with Yao *et al*^[27] who failed to demonstrate a significant rise of GSH in livers of rats treated with NAC.

In the present study, glutathione used in scavenging hydroxyl radicals stimulated glutathione reductase activity, thereby maintaining nearly normal GSH. NAC failed to decrease glutathione reductase activity in RMP-exposed animals. These results might be due to stimulation of glutathione reductase activity by the low level of glutathione and or a negative feedback inhibition by the normalization of GSH levels in NAC treated animals.

GST activity against EA was stimulated in animals treated with RMP and RMP+NAC. GST is specific for removal of peroxidation products and induction of this enzyme might be in response to lipid peroxidation so as to remove the highly toxic materials from cellular milieu. Adachi *et al*^[28] reported that RMP can induce GST activity in rats.

The histopathological patterns of liver injury in the present study are similar to the earlier findings^[9, 29]. Sodhi *et al*^[5] also showed that patchy necrosis occurs in RMP-treated animals. In the present study, NAC failed to protect completely against RMP-induced hepatic injuries but it protects completely against hepatic injuries in rats after treatment with INH+RMP^[30].

In conclusion, RMP exposure to animals does alter the profile of antioxidant enzymes while non-protein thiol status can be well preserved. The protective effect of NAC on RMP-induced hepatic injury might be due to prevention of lipid peroxidation as well as decline in superoxide dismutase in animals exposed to RMP and NAC. However, partial hepatotoxicity due to RMP might be due to some other mechanisms of injury.

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RAPID COMMUNICATION

Correlation between *Saccharomyces cerevisiae* DNA in intestinal mucosal samples and anti-*Saccharomyces cerevisiae* antibodies in serum of patients with IBD

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remain relatively stable thereafter in immunological susceptible persons.

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Abstract

AIM: To investigate the correlation between ASCA and presence of mucosal *S. cerevisiae* DNA in a population of CD, ulcerative colitis (UC) patients and controls.

METHODS: *S. cerevisiae*-specific primers and a fluorescent probe were designed for a 5' exonuclease real time PCR (TaqMan™) assay, which is a homogenous system using a fluorescent-labelled probe for the detection of PCR product in real time. We analyzed the relation of the PCR results with the ASCA findings in a group of 76 inflammatory bowel disease (IBD) patients (31 CD, 45 UC) and 22 healthy controls (HC).

RESULTS: ASCA (*IgA* or *IgG*) were positive in 19 (61%) patients with CD, 12 (27%) with UC and none of the HC. PCR amplification was inhibited and excluded from the final results in 10 (22%) UC patients, 7 (22%) CD patients, and 6 (30%) HC. In only 15 of the mucosal samples, *S. cerevisiae* DNA was detected by real time PCR, including 7 (29%) in CD, 7 (19%) in UC, 1 (6%) in HC. In 4 CD and in 4 UC patients, ASCA and mucosal *S. cerevisiae* were positive. Mucosal *S. cerevisiae* was present in combination with negative ASCA *IgA* and *IgG* in 3 UC, and 3 CD patients.

CONCLUSION: We conclude that since the presence of *S. cerevisiae* in colonic mucosal biopsy specimens is very rare, ASCA is unlikely to be explained by continuous exposure to *S. cerevisiae* in the mucosa. Therefore, ASCA formation must occur earlier in life and levels

INTRODUCTION

Inflammatory bowel disease (IBD) is characterized by a chronic relapsing intestinal inflammation. The causes of IBD are unknown but genetic, environmental, immunological, and microbial factors may be involved. Results from studies on animals suggest that the intestinal flora participates in the initiation and perpetuation of IBD^[1].

Saccharomyces cerevisiae is the most common species of the genus *Saccharomyces*. It is used in baker's yeast and readily finds its way into our foods^[2]. Several case-series describe serious *S. cerevisiae* infections in immune-compromised patients, usually following treatment with broad-spectrum antibiotics^[3-6]. Opportunistic infections by viral and fungal agents have been described as occurring in rare cases of ulcerative colitis (UC). Only one case describes diarrhea associated with *S. cerevisiae* cultured in the stool specimen of an UC patient^[7]. So far, no studies have been published concerning the presence of *S. cerevisiae* in the intestinal tissue of patients with IBD. *S. cerevisiae* specific primers and a fluorescent probe were designed for a 5' exonuclease real time PCR (TaqMan™) assay. This method provides high specificity and sensitivity for detecting *S. cerevisiae* DNA but is hampered by the difficulties arising from DNA extraction of *Saccharomyces* species.

In 1988, the presence of anti-*Saccharomyces cerevisiae* antibodies (ASCA) in patients with Crohn's disease (CD) was firstly described^[8]. The ASCA test for diagnosing CD has a sensitivity of 72% and a specificity of 82%^[9-12]. The underlying cause of generating *S. cerevisiae* antigens

supporting the specific antibody response in CD is still unknown. ASCA are thought to result from a specific antibody response to the *S. cerevisiae* cell wall mannan (phosphopeptidomannans). It is unknown whether this is a direct response towards the yeast itself or an epiphenomena with a similar immunologic response towards another antigen. It is postulated that the yeast wall cell mannan may mimic a high mannose-containing molecule towards which the antibody is directed inducing a hypersensitivity reaction during inflammation^[12,13].

MATERIALS AND METHODS

Patients

Seventy-six patients with IBD (45 UC, 31 CD) and 22 healthy age- and sex-matched controls regularly visiting the Departments of Gastroenterology from the VU University Medical Center, Amsterdam, the Netherlands, a third line referral center, and St Anna Hospital, Geldrop, the Netherlands, a regional hospital, were enrolled in the study. IBD patients with clinical complaints compatible with active inflammation of the mucosa were screened for the study, but both IBD patients with active inflammation as well as quiescent disease assessed by macroscopic endoscopic findings were included in the study. The diagnosis of CD and UC was based on standard endoscopic, histological, and radiographic features^[14]. Disease localization and activity, demographical data and medication were documented. Fifty-four percent of UC patients and 42% of CD patients was treated with immunosuppressive medication (Table 1). None of the patients was treated with probiotics containing *S. boulardii*. However, they were not requested to use a diet low of *S. cerevisiae* either.

During sigmoidoscopy, 2 mucosal biopsy specimens were obtained from the sigmoid and directly snap frozen in liquid nitrogen and then stored at -18°C until further analysis. In addition, blood samples were drawn for detection of ASCA antibodies.

This study was approved by the Medical Ethical Committee of the VU University Medical Center, Amsterdam, the Netherlands.

ASCA ELISA

ASCA IgA and IgG were determined by commercially available ELISA kits (Inova, Uniprom Diagnostics BV, Krimpen aan de IJssel, the Netherlands). The antigen consists of phosphopeptidomannan (PPM) extracted from *S. cerevisiae*. ASCA ELISAs were performed according to the manufacturer's instructions. ASCA results were expressed as arbitrary units with a cut-off for positivity of 25 U/mL as advised by the manufacturer and described elsewhere^[15]. Serum was considered positive if either IgA or IgG or both were positive. Serum was considered negative if both IgA and IgG ASCA were negative.

DNA isolation

Biopsy specimens were pretreated with 200 µL of 5 g/L collagenase A (Roche Diagnostics, Almere, Nederland B.V.) and incubated at 37°C overnight with continuous shaking. Subsequently, each biopsy was divided into two equal cell

fractions. One fraction was spiked with 20 colony forming units/µL *S. cerevisiae*. Both fractions were incubated for 90 min at 37°C with 600 µL of sorbitol buffer and 200 U of lyticase (Sigma-Aldrich, Steinheim, Germany), prior to the isolation of chromosomal DNA with the DNeasy™ Tissue Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The end volume after the extraction of the DNA from the biopsy specimen was 100 µL. The control fraction of the biopsy specimen contained 20 CFU equivalents/µL (elution concentration).

S. cerevisiae specific primers and a fluorescent probe for a 5' exonuclease RT PCR (TaqMan™) assay were designed with the Primer Express software package (Applied Biosystems, Foster City, CA, USA). This assay is a homogeneous system with a fluorescent double-labeled probe for the quantitative detection of PCR product. This provides a rapid automated combined PCR amplification and detection system with no post-amplification manipulation of amplicons, thereby considerably reducing the risk of contamination. Coupled with a quick, simple DNA extraction method, this protocol allows for rapid specification of clinical isolates. Amplification reactions were carried out in a total volume of 25 µL. Reaction mixtures contained 1x universal master mix (Applied Biosystems), 300 nmol of *Saccharomyces* forward primer (5'-GAA ATG CCA CCG TGA ATG C), 300 nmol of *Saccharomyces* reverse primer (5'-CTT TGG TGG TGA TCC TCT ATG ATT G) and 100 nmol of labeled probe (FAM - TGG CAC CAT GAA CCC TAG CGT CGT T - TAMRA), and 5 µL of DNA. To prevent inhibition, 5 g/L bovine serum albumin (BSA) (Sigma-Aldrich, Steinheim, Germany) was added to the PCR mixtures. Amplification was carried out after an incubation for 2 min at 50°C and for 10 min at 95°C, followed by 45 cycles at 95°C for 15 s, and at 60°C for 1 min. These reactions were performed on an ABI Prism 7000 Sequence Detection System (SDS) (Applied Biosystems, Figure 1).

Statistics

SDS software was used for analysis of the data obtained from RT-PCR results, and *t*-test with separate variance estimates were used to test differences between patients with CD, UC and controls.

RESULTS

ASCA (IgA or IgG) were identified in 19 (61%) CD patients, 12 (27%) UC patients and none of the HC. Of the patients, 42% of CD patients and 54% of UC patients were using immunosuppressive medication (Table 2).

To determine the sensitivity of real time PCR, *S. cerevisiae* cells were cultured and the amount of colony forming units (CFU) were counted. After DNA isolation a dynamic range, based on the related amount of DNA (CFU equivalents) was made from 10 to 10⁵ (Figure 2).

Specificity of the PCR for *S. cerevisiae* was examined by comparing the results of pure *S. cerevisiae* DNA and *S. cerevisiae* DNA mixed with DNA isolated from 10 different bacteria (*Bacteroides vulgatus*, *Bifidobacterium adolescentis*, *Clostridium difficile*, *Lactobacillus acidophilus*, *Propionibacterium acnes*, *Actinomyces viscosus*, *Enterococcus faecalis*, *Fusobacterium*

Table 1 Medication at time of harvest of biopsy specimens

| | Medication | No of patients (%) |
|---------------------------|---------------------------|--------------------|
| UC | Prednisone | 12/37 32 |
| | AZA | 11/37 30 |
| | ASA | 8/37 22 |
| | Ciclosporine | 4/37 11 |
| | No medication | 12/37 32 |
| | Any immunomodulating drug | 20/37 54 |
| CD | Prednisone | 6/24 25 |
| | AZA | 5/24 21 |
| | ASA | 2/24 8 |
| | Ciproxin | 1/24 4 |
| | Anti-TNF | 2/24 8 |
| | No medication | 13/24 54 |
| Any immunomodulating drug | 10/24 42 | |

Table 3 ASCA and RT-PCR detection of *S. cerevisiae* in CD patients

| | ASCA positive | ASCA negative | Total |
|-----------------|---------------|---------------|-------|
| RT-PCR positive | 4 | 3 | 7 |
| RT-PCR negative | 12 | 5 | 17 |
| Total | 16 | 8 | 24 |

Table 4 ASCA and RT-PCR detection of *S. cerevisiae* in UC patients

| | ASCA positive | ASCA negative | Total |
|-----------------|---------------|---------------|-------|
| RT-PCR positive | 4 | 3 | 7 |
| RT-PCR negative | 8 | 22 | 30 |
| Total | 12 | 25 | 37 |

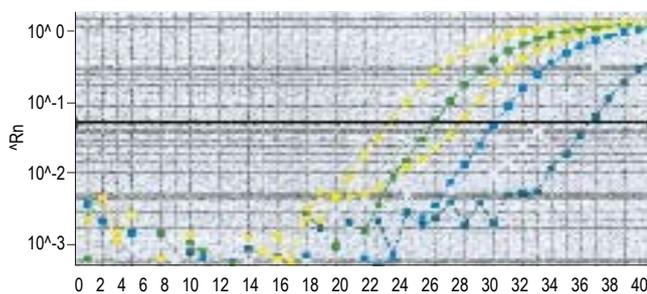


Figure 1 Annealing cycles of *S. cerevisiae*

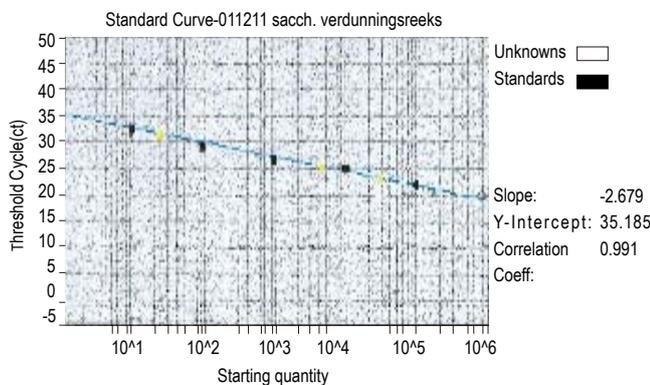


Figure 2 Dilution series of *S. cerevisiae*

nucleatum, *Escherichia coli*, *Streptococcus pyogenus*, *Listeria monocytogenes*, *Listeria ivanovi*), belonging to the normal gut flora and 6 different *Saccharomyces* species (*S. boulardii*, *S. unisporus*, *S. kluyveri*, *S. pastorianus*, *S. paradoxus*, *S. servazzi*). In addition, this DNA mixture neither influenced the sensitivity nor the specificity of the *S. cerevisiae* amplification (data not shown). Among the 15 mucosal biopsies, *S. cerevisiae* DNA was detected in 7 (29%) CD patients, 7 (19%) UC patients, 1 (6%) HC. The amount of *S. cerevisiae* DNA was low and varied. Positive values were to be found between 37-38 CFU equivalents. Inhibition during amplification occurred in 8 (18%) UC patients, 7 (22%) CD patients and 6 (30%) HC.

Furthermore, 25% (4/16) CD patients and 33% (4/12) UC patients showed positivity for both ASCA and mu-

cosal *S. cerevisiae* DNA. In 12% (3/25) UC and 38% (3/8) CD patients, mucosal *S. cerevisiae* DNA was present in combination with negative ASCA *IgA* and *IgG* (Tables 3 and 4).

DISCUSSION

ASCA (*IgA* or *IgG*) were positive in 19 (61%) CD patients, 12 (27%) UC patients and none of the HC. Out of the 15 mucosal biopsies, RT PCR demonstrated the presence of *S. cerevisiae* DNA in 7 (29%) CD patients, 7 (19%) UC patients, and 1 (6%) HC. No significant correlation could be found between ASCA and the presence of *S. cerevisiae* DNA isolated from mucosal biopsies.

Frequencies of ASCA reported in literatures range from 39-76% for CD patients, 5-15% for UC patients to 0-5 % in controls^[8, 11, 12, 16-23]. In this study ASCA positivity in the UC group was high (27%) as compared to earlier ASCA studies from our group and reported frequencies in literature.

The presence of ASCA appears not to be related to disease activity^[23]. Therefore, we assume that our selection of patients (clinical complaints compatible with active disease) is not likely to influence ASCA status or the presence of *S. cerevisiae*. Forty-two percent of CD patients and 54% of UC patients were using immunosuppressive agents in this cohort (Table 1). This high percentage of patients using immunosuppressive agents reflects the overall disease activity of our patient population, being relatively severe. However, use of immunosuppressive agents does not explain the high frequency of ASCA in our UC population since most ASCA-positive UC patients did not use this type of medication. Therefore, it can be hypothesized that healing of the mucosa with the use of immunosuppressive agents will lead to decreasing ASCA levels, as has been reported in the treatment of children with active CD^[22]. ASCA were detected with well-validated commercially available kits that have been described in previous studies^[9].

Mucosal permeability appears increased in active CD as a consequence of direct effects of pro-inflammatory molecules and transmigrating neutrophils. However, considerable controversy exists regarding a primary genetically

Table 2 RT-PCR and ASCA results in relation to medication

| | Medication | RT-PCR + (%) | RT-PCR - (%) | ASCA + (%) | ASCA - (%) |
|----|---------------|--------------|--------------|------------|------------|
| UC | Prednisone | 6/7 86 | 6/30 20 | 5/12 42 | 7/25 28 |
| | AZA | 1/7 14 | 10/30 33 | 2/12 17 | 9/25 36 |
| | ASA | 3/7 43 | 5/30 17 | 0 | 8/25 32 |
| | Ciclosporine | 1/7 14 | 3/30 10 | 1/12 8 | 3/25 12 |
| | No medication | 0 | 12/30 40 | 7/12 58 | 6/25 24 |
| CD | Prednisone | 2/7 29 | 4/17 24 | 4/16 25 | 2/8 25 |
| | AZA | 2/7 29 | 3/17 18 | 2/16 13 | 3/8 38 |
| | ASA | 1/7 14 | 1/17 6 | 1/16 6 | 1/8 13 |
| | Ciproxin | 1/7 14 | 0 | 1/16 6 | 0 |
| | Anti-TNF | 2/7 29 | 0 | 2/16 13 | 0 |
| | No medication | 2/7 29 | 11/17 65 | 8/16 50 | 5/8 63 |

determined defect in epithelial barrier function^[24-26].

In a small study^[27], intestinal permeability, tested by a cellobiose/mannitol test, was raised in 6 (37%) of the CD patients and in 11% of their relatives. This altered intestinal permeability was unrelated to sex, age, disease activity, localization, duration, treatment schedule, as well as to serum ASCA positivity, a rather stringent subdivision in such a small patient group. Although an interesting finding, this makes interpretation difficult. Furthermore, genetic polymorphisms were not taken into account.

ASCA is not related to mucosal disintegrity, because ASCA is independent of disease activity^[28]. Elevated serum levels of ASCA did not primarily seem to result from a defect of the gut barrier^[29]. These data fit in with the hypothesis that a relationship between antibody responses toward microbial antigens and complicated small bowel diseases reflect the interplay between a genetically susceptible host and relevant luminal flora, as has been suggested before, relating intestinal permeability to environmental factors, and ASCA generation to genetic predisposition^[23].

To our knowledge, this is the first study describing the presence of *S. cerevisiae* DNA in intestinal mucosa of IBD patients by using RT-PCR. The presence of ASCA antibodies could not be correlated with mucosal *S. cerevisiae* DNA in biopsy specimens taken during sigmoidoscopy although the RT-PCR test is highly sensitive and specific. Every individual sample was checked for inhibition by a simultaneous spiked amplification. Inhibition during amplification occurred in 8 (18%) UC patients, 7 (22%) CD patients and 6 (30%) HC. Extraction of DNA from yeast requires special enzymes to remove the cell wall^[30]. Theoretically, excluding a number of patients from the total patients due to inhibition of the RT-PCR amplification may influence the results, particularly taking into account the relatively small study population. However, amplification problems were equally distributed in all groups. Furthermore, one could hypothesize that the presence of *S. cerevisiae* is higher in ileal mucosa than in the sigmoid since higher ASCA levels were detected in CD patients with ileal localization of disease^[28]. Preliminary RT-PCR data from our group show comparable numbers of *S. cerevisiae* in the left and right side of the colon consistent with an equally distributed presence of *S. cerevisiae* throughout the (distal) intestinal tract (Akol, personal communication). Interbiopsy variability between multiple biopsies taken at the same

localization was low. Therefore, our results were unlikely to be biased by neither the number of biopsy specimens nor the localization from where the biopsies were obtained.

The presence of mucosal *S. cerevisiae* DNA with concomitant negative serum ASCA IgA and IgG in one UC and two CD patients can not be explained by the intestinal leakage hypothesis. This finding corroborates the hypothesis that ASCA formation is an epiphenomenon (in genetically susceptible individuals) rather than the result of antigenic challenge by intestinal presence of *S. cerevisiae* in leaking mucosa. On the contrary, we postulate that since the presence of *S. cerevisiae* DNA in mucosal biopsy specimens is very rare, presence of ASCA can not be explained by antigenic exposure to *S. cerevisiae*.

Another hypothesis is that everyone encounters *S. cerevisiae* early in life. In some patients, the (transitory) *S. cerevisiae* presence may lead to ASCA formation, particularly in people prone to develop CD. Only in a small percentage of people, life-long colonisation may be the result, comparable to what has been documented in other species such as *Clostridium difficile*. Although intra-individual, longitudinal determination values of ASCA were not tested in this study, ASCA formation may be initiated early in life and perpetuate thereafter as described in adult populations^[31]. In contrast, ASCA levels decrease after treating active inflammation in children with CD^[22,32,33]. These differences between stable ASCA values in an adult population and decreasing ASCA values in a paediatric population have recently been described for a population with coeliac disease^[34], pointing at differences in antigen handling/immunologic response in children and adults.

In conclusion, our study demonstrates no any correlation between the presence of ASCA antibodies and mucosal *S. cerevisiae* DNA. Although the study population was small, this finding underscores the hypothesis that ASCA antibodies are not solely formed as a reaction on the mannan from the yeast cell wall but rather is an epiphenomenon with a similar immunologic response towards another, yet unidentified antigen.

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RAPID COMMUNICATION

Particularly interesting new cysteine-histidine rich protein expression in colorectal adenocarcinomas

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CONCLUSION: The expression of PINCH was upregulated in colorectal cancers, and especially at the margin of tumours, and further was related to mucinous and signet-ring cell carcinomas. The results suggest that expression of PINCH may be involved in the tumourigenesis and aggressiveness of colorectal cancers.

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Key words: PINCH; Stroma; Colorectal cancer; Immunohistochemistry

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Abstract

AIM: To study the relationship between particularly interesting new cysteine-histidine rich protein (PINCH) expression and clinicopathological factors in Chinese colorectal cancer patients.

METHODS: The expression of PINCH was examined by immunohistochemistry in 141 samples of primary colorectal adenocarcinoma and 92 normal samples of colorectal mucosa. Eighty of the cases had both primary tumour and normal mucosa from the same patients.

RESULTS: PINCH was expressed in the stroma of normal mucosa and tumours. PINCH expression in tumour-associated stroma was increased compared to normal mucosa in both unmatched cases ($n = 141$, $X^2 = 85.79$, $df = 3$, $P < 0.0001$) and matched cases ($n = 80$, $X^2 = 45.86$, $df = 3$, $P < 0.0001$). Among 135 tumours with visible invasive margin, 86 (64%) showed stronger PINCH expression at the invasive margin than in the intratumoural stroma. The frequency of PINCH strong expression in mucinous and signet-ring cell carcinomas was higher (52%) compared to non-mucinous carcinomas (29%, $X^2 = 5.13$, $P = 0.02$). We did not find that PINCH expression was related to patient's gender, age, tumour location, tumour size, gross status, histological type, differentiation, invasion depth, lymph node status and Dukes' stage ($P > 0.05$).

INTRODUCTION

Studies have shown the importance of stromal tissue in regulating the physiological processes of the body. Disruption of stromal-epithelial interactions and cell adhesion alters cellular signalling, which influences proliferation, angiogenesis, differentiation, motility, death, genomic integrity and other phenotype in the cells and tissues^[1-3]. Extra-cellular matrix (ECM) adhesion is a fundamental process that controls a variety of cellular processes including cell shape changes and migration. Cell-ECM interactions are mediated by a selective group of membrane and cytoplasmic proteins at the ECM contact sites. Integrin-linked kinase (ILK) is a multidomain protein that plays an important role at ECM adhesion sites^[2-7].

PINCH is a LIM domain adapter protein. The PINCH gene is located on chromosome 2q12.2. PINCH and ILK components function as an adaptor protein connecting the growth factor-signalling pathways with the integrin-signalling pathway. It is found in focal adhesions, large cellular complexes that link extracellular matrix to the actin cytoskeleton interacting with ILK and Nck2. PINCH has been implicated as a platform for multiple protein-protein interactions mediating integrin signalling within focal adhesions^[4, 7-11].

In the present study, the expression of PINCH was immunohistochemically studied in 141 samples of primary colorectal adenocarcinoma and 92 normal mucosa samples. The aims were to investigate the expression of PINCH in

normal mucosa and primary tumours in Chinese colorectal cancer patients and to identify the relationship between PINCH expression and clinicopathological variables including patient's gender, age, tumour location, tumour size, gross status, histological type, grade of differentiation, invasive depth, metastasis in the lymph nodes and Dukes' stages.

MATERIALS AND METHODS

Patients

Tumour samples were collected from 141 patients with colorectal adenocarcinoma diagnosed at Department of Pathology, Tangshan Worker's Hospital, China, between 2000 and 2002. The study also included 92 normal mucosa samples, 80 of which were matched with the tumours, i.e., from the same patients. Normal samples were taken from the margin of the distant resection being histologically free from pre-tumour and tumour. None of the patients received preoperative radiotherapy or chemotherapy. The patient's gender, age, tumour location, tumour size, gross status, histological type, grade of differentiation, invasive depth, lymph node status and Dukes' stages were obtained from surgical and pathological records. The mean age of the patients was 56 years (range, 30-80 years). Tumours of the caecum, ascending and transverse colon were defined as proximal tumours and tumours of the descending and sigmoid colon and rectum were defined as distal tumours. The mean tumour size was 4.9 cm (range, 0.8-20 cm). Tumours were graded as better (well + moderate) and worse (poor) differentiation. All samples were examined by two pathologists.

Immunohistological staining and evaluation

The preparation, specificity and reliability of the rabbit polyclonal PINCH antibody (obtained kindly from Professor Ann Rearden, Department of Pathology, University of California, La Jolla, CA) used in this study have been described previously^[10,11].

The paraffin-embedded tissue sections (5µm) were deparaffinised in xylene and rehydrated in graded ethanol. In order to expose masked epitopes, the sections were boiled in 0.01 mol/L Tris-EDTA buffer (pH 9.0) in a high pressure-cooker, kept at room temperature for 30 min and washed with phosphate-buffered saline (PBS, pH 7.4). The activity of endogenous peroxidase was blocked in 0.5% H₂O₂ in methanol for 10 min and washed with PBS. The sections were incubated with the 2µg/mL primary PINCH antibody at 4 °C overnight and washed with PBS. The sections were incubated with polymer enhancer for 20 min, then with polymerized HRP-anti mouse/rabbit IgG antibody for 30 min and rinsed in PBS between the incubation steps. After washed with PBS, peroxidase reaction was performed by use of 3,3'-diaminobenzidine for 5 min [Elivision TM plus polyer HRP (mouse/rabbit) IHC kit, Fuzhon Maixin Biology Technology Limited Company, Fuzhou, China]. After counterstained with haematoxylin, the sections were dehydrated and mounted. Normal mucosa and the matched primary tumours were stained in the same run of immunostaining to avoid bias on the pattern and intensity of the staining. Sections known to show

Table 1 PINCH expression in relation to clinicopathological variables in colorectal cancers

| Variables | n | Strong PINCH n (%) | Weak PINCH n (%) | P |
|---------------------------|-----|-----------------------|---------------------|------|
| Gender | | | | 0.67 |
| Male | 71 | 23 (32) | 48 (68) | |
| Female | 70 | 25 (36) | 45 (64) | |
| Age (years) | | | | 0.77 |
| ≤56 | 70 | 23 (33) | 47 (67) | |
| >56 | 71 | 25 (35) | 46 (65) | |
| Tumour location | | | | 0.61 |
| Proximal | 42 | 13 (31) | 29 (69) | |
| Distal | 99 | 35 (35) | 64 (65) | |
| Tumour size (cm) | | | | 0.64 |
| ≤4.9 | 86 | 28 (33) | 58 (67) | |
| >4.9 | 55 | 20 (36) | 35 (64) | |
| Gross status | | | | 0.60 |
| Ulcerated | 108 | 38 (35) | 70 (65) | |
| Polypoid/Lager fungating | 33 | 10 (30) | 23 (70) | |
| Histological type | | | | 0.02 |
| Non-mucinous | 117 | 34 (29) | 83 (71) | |
| Mucinous/signet-ring cell | 27 | 14 (52) | 13 (48) | |
| Grade of differentiation | | | | 0.59 |
| Better | 99 | 33 (33) | 66 (67) | |
| Worse | 42 | 16 (38) | 26 (62) | |
| Lymph node status | | | | 0.11 |
| Non-metastasis | 100 | 30 (30) | 70 (70) | |
| Metastasis | 41 | 18 (44) | 23 (56) | |
| Invasive depth | | | | 0.48 |
| Intra-bowel wall | 105 | 34 (32) | 71 (68) | |
| Ultra-bowel wall | 36 | 14 (39) | 22 (61) | |
| Dukes' stage | | | | 0.17 |
| A | 52 | 12 (23) | 40 (77) | |
| B | 51 | 19 (37) | 32 (63) | |
| C | 34 | 15 (44) | 19 (56) | |
| D | 4 | 2 (50) | 2 (50) | |

strong immunostaining for PINCH were used in each run receiving either the primary antibody or PBS as positive and negative controls. In all the staining procedures, the positive controls showed staining clearly and there was no staining in the negative controls.

PINCH immunostaining was independently examined by two pathologists in a blinded fashion without knowledge of the clinical and pathological information. To avoid artificial effect, the staining on the margins of sections and areas of poorly presented morphology were not counted. The intensity of PINCH staining in stroma was graded as negative (no positive cells), weak (<5% positive cells), moderate or strong staining. In cases with discrepant results, a consensus score was reached after re-examination.

Statistical analysis

The chi square method was used to test the relationship between the frequencies of PINCH expression in normal mucosa and tumour as well as between PINCH expression in tumour and clinicopathological variables. All P values cited were two-sided and $P < 0.05$ was considered statistically significant.

RESULTS

PINCH was expressed in the stroma in both normal

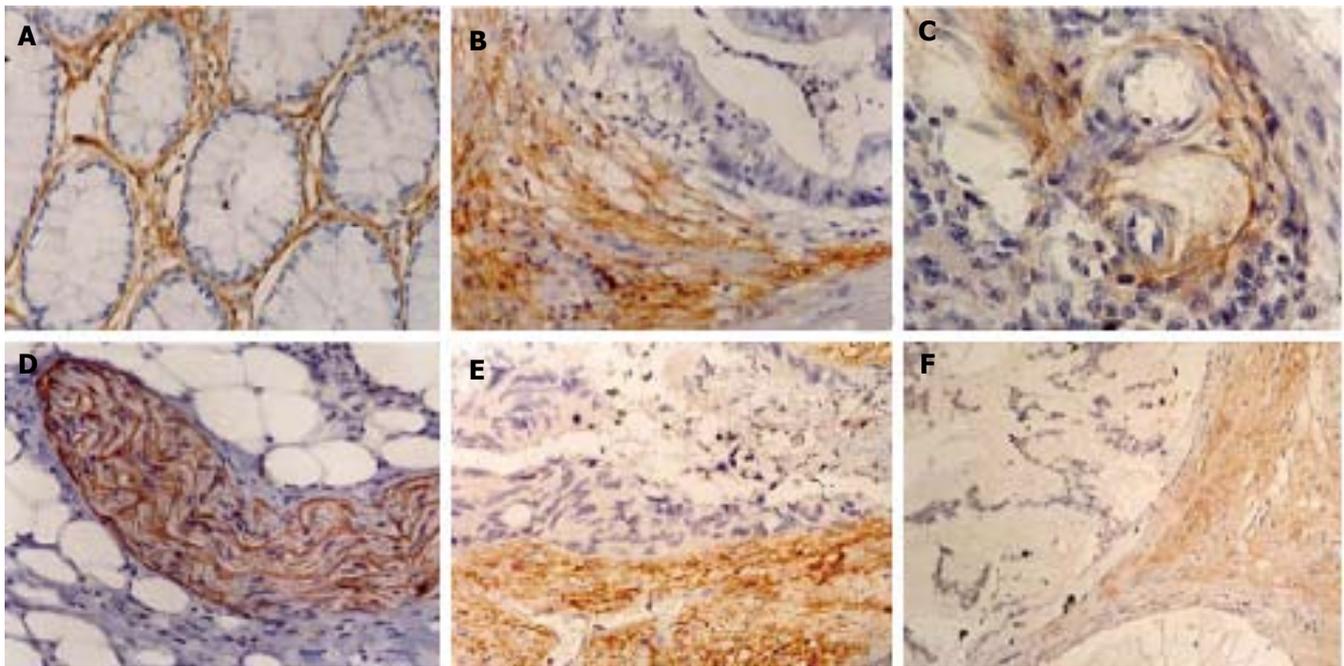


Figure 1 PINCH immunohistochemical staining in normal mucosa (A), adenocarcinoma (B), tunica adventitia of blood vessel (C), peripheral nerve fascicles (D) and stronger expression of PINCH at the invasion edge than in the inner area (E), and at the invasion edge of mucinous carcinoma (F).

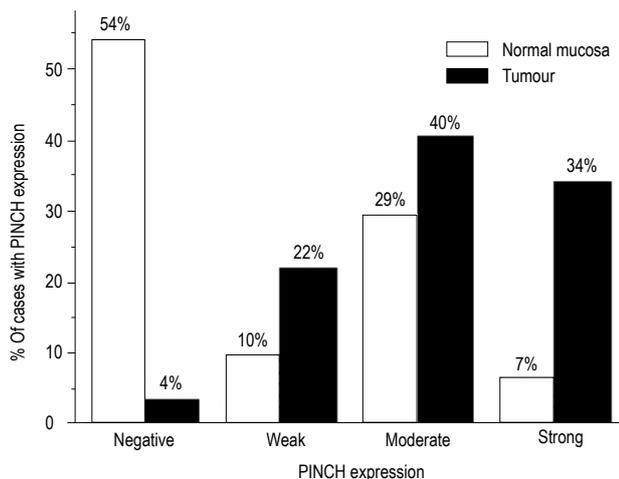


Figure 2 Frequency of PINCH immunohistochemical staining in 92 normal mucosa and 141 tumour samples.

mucosa and tumour samples, mainly in cytoplasm of fibroblasts and myofibroblasts, a proportion of endothelial cells in the tumour vasculature and peripheral nerves (Figure 1). Among 135 tumours with visible margin, 86 (64%) showed stronger PINCH expression at the invasive edges than in the intratumoural stroma, 31 (23%) showed opposite evidence, the remained 18 (13%) showed the same staining intensity. There was no PINCH expression in normal epithelial and tumour cells (Figure 1).

PINCH expression was negative in 50 (54%), weak in 9 (10%), moderate in 27 (29%) and strong in 6 (7%) in 92 normal mucosa samples, and in 5 (4%), 31 (22%), 57 (40%) and 48 (34%) respectively in 141 tumour samples. The expression was significantly increased in tumour samples compared to normal mucosa samples ($\chi^2 = 85.79$, $df = 3$,

$P < 0.0001$, Figure 2). Even in the 80 matched cases of normal mucosa samples (51%, 10%, 33% and 6%) and tumours (5%, 20%, 43% and 33%), the significance was still remained ($\chi^2 = 45.86$, $df = 3$, $P < 0.0001$).

Since the clinicopathological features of tumours with negative, weak and moderate staining were similar, they were combined as one group (weak group) to compare with strong group in statistical analyses. Table 1 summarises the expression of PINCH in relation to patient's gender, age, tumour location, tumour size, gross status, histological type, grade of differentiation, invasive depth, lymph node status and Dukes' stage. The result showed that the frequency of strong PINCH expression was higher in mucinous/signet-ring cell carcinomas (52%) than in non-mucinous carcinomas (29%, $\chi^2 = 5.13$, $P = 0.02$, Table 1). Figure 1 shows a mucinous carcinoma with PINCH expression. The frequency of strong PINCH expression in Dukes C+C+D tumour (45%) tended to be higher than that in Dukes' A+B tumour (30%, $\chi^2 = 2.65$, $P = 0.10$). It was also shown that patients with lymph node metastasis seemed to have higher PINCH expression (44%) than those without metastasis (30%, $\chi^2 = 2.50$, $P = 0.11$). We did not find other relationships between the expression of PINCH and clinicopathological variables ($P > 0.05$, Table 1).

DISCUSSION

In the present study, we observed that PINCH presented in fibroblasts, myofibroblasts, a proportion of endothelial cells of the tumour vasculature and peripheral nerves. The expression of PINCH was especially strong in stroma at the invasive edges of tumours compared to the intratumoural stroma, suggesting that PINCH as a biological factor, may be involved in the angiogenesis and invasiveness of tumour. This evidence may partly

explain why strong PINCH expression is associated with a poor prognosis in colorectal cancer patients^[12]. In the present study, the frequency of strong PINCH expression was significantly higher in mucinous and signet-ring cell carcinomas than in non-mucinous carcinomas, which may also explain why PINCH expression is related to a poor clinical outcome. Studies demonstrated that patients with mucinous colorectal carcinomas have a worse prognosis than those with non-mucinous carcinomas^[13-15], indicating that mucins interfere with immunologic recognition of tumour cells by masking antigenic epitopes with sialic acid residues and inhibiting lymphocyte infiltration^[16]. We have previously reported that there is less inflammatory infiltration in colorectal cancer with strong PINCH expression^[12].

PINCH is directly associated with ILK and Nck-2 proteins that are downstream effectors of integrin and growth factor signalling. Some of these growth factors, such as PDGF-mediated tumour-stromal interactions are important to tumour growth. PINCH is required for ILK localisation to integrin-containing adherens junctions where ILK regulates fibronectin matrix assembly^[4, 6, 7, 9-12], suggesting that PINCH protein may increase the upregulated growth factor signalling in stromal cells and a marker for stroma angiogenesis and invasion of tumour cells.

PINCH protein is involved in integrin-mediated cell-ECM interactions, where the different mechanisms or different genetic pathways may develop different histological types of tumour by specific classes of carcinogens. In this context, the expression of PINCH may be associated with the phenotype of epithelial cells in the colorectum. The frequency of K-ras mutation and microsatellite instability is higher in mucinous carcinomas than in non-mucinous carcinomas^[17-20]. In contrast, mucinous carcinomas exhibit significantly less p53 mutation and protein^[20-23]. These results lead to the hypothesis that K-ras and microsatellite instability may influence mucus production or degradation, resulting in the development of mucinous carcinoma. In contrast to non-mucinous tumours, the development of mucinous carcinomas may be independent from p53 alteration. Thus, PINCH may be another factor involved in the development of mucinous carcinomas.

In conclusion, the expression of PINCH was upregulated in colorectal cancers, and especially at the margin of the tumours, and further was related to mucinous carcinomas. The results suggest that expression of PINCH may be involved in the tumorigenesis and aggressiveness of colorectal cancers.

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RAPID COMMUNICATION

Involvement of lymphocytes in dextran sulfate sodium-induced experimental colitis

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Abstract

AIM: To investigate the roles of lymphocytes in the development of dextran sulfate sodium-induced colitis.

METHODS: Using various doses of dextran sulfate sodium (DSS), we induced colitis in wild-type B6 control and Rag-1 knockout (H-2^b haplotype) mice, and evaluated the colitis in terms of symptomatic and histologic parameters, such as weight loss, survival, severity of diarrhea, shortage of colon length and histological changes. Symptomatic parameters were checked daily and histological changes were scored.

RESULTS: Although development of colitis in Rag-1 knockout mice treated with high dose (5%) of DSS was comparable to that in B6 control mice, colitis progression was much more tolerable in Rag-1 knockout mice compared to than in B6 mice treated with low dose (1.5%) DSS. Symptomatic parameters as well as histopathologic changes were improved in Rag-1 knockout mice.

CONCLUSION: These results indicate that the presence of lymphocytes contributes to colitis progression at low dose of DSS stimulation. Lymphocytes may play roles as an aggravating factor in DSS-induced colitis.

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Key words: Dextran sulfate sodium; Colitis; Lymphocyte; Rag-1; Knockout

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INTRODUCTION

Despite many years of researches, the pathogenesis of human inflammatory bowel disease (IBD) remains poorly understood^[1]. Clinical studies are limited to the patients with established disease; therefore, early events of the disease are difficult to approach. Several experimental models of colitis have been introduced to study factors that can induce chronic inflammation and investigate the evolution of colitis from the initial pathologic event to its final clinical manifestation. One of these models is based on the oral administration of dextran sulfate sodium (DSS) in the drinking water of mice, which results in an acute and chronic colitis with some morphological changes similar to human ulcerative colitis^[2]. In addition, this model has been shown to respond to the anti-colitis drugs, such as sulfasalazine, olsalazine and mesalazine, that are used in human ulcerative colitis and have several immunomodulating functions^[3].

The pathogenic mechanism of this colitis is attributed to its direct toxic effects to colonic epithelial cells^[4], namely their relative independence from lymphocyte-mediated responses. Thus, in DSS-induced colitis, it is reported that mice lacking T cells, B cells, and NK cells can still develop colitis in response to DSS^[5]. However, some studies showed the contradictory results, which suggest that lymphocytes play roles in the development of DSS-induced colitis. In acute stages of DSS-induced colitis, T cell response consists of polarized Th1 response, but in later and more chronic phase of inflammation, a mixed Th1/Th2 response occurs^[6]. NKT cells, in contrast to the above CD4⁺ T cells, were reported to play a protective role in DSS-induced colitis^[7].

In this study, we investigated the role of lymphocytes in the development of DSS-induced colitis using Rag-1 knockout (KO) mice and found that the severities of the DSS-induced colitis were dramatically decreased in Rag-1 KO mice.

MATERIALS AND METHODS

Animals

Eight-week-old male C57BL/6 (B6, H-2^b-haplotype) and

Rag-1 knockout (Rag-1 KO, H-2^b-haplotype) mice were purchased from Orient (Chunduk, Korea) and Jackson laboratory (Bar Harbor, ME, USA), respectively. The mice housed in a specific pathogen-free condition at Hallym University (Chuncheon, Korea) for at least 6 weeks for adaptation to the environmental changes. All mice used in this study were 14 and 16 weeks of age. All experimental animals were cared for, maintained, and terminated in accordance with the Hallym University Guidelines.

Colitis model

Colitis was induced by addition of 1.5% DSS (M.W. 36,000-50,000; MP Biomedicals, Irvine, CA) to drinking water for 15 d. Animals were monitored daily for loss of body weight, diarrhea, and survival for 15 d. The severity of diarrhea was evaluated as the following scores^[8]: no diarrhea = 0; mild diarrhea = 2; severe watery diarrhea = 3; mild diarrhea with blood = 4; and severe watery diarrhea with blood = 8. At 15 d or when mice had lost more than 20% of their initial body weight after DSS administration, they were sacrificed.

Histopathological analysis

Colons were removed, fixed in 100 mL/L neutral formalin, embedded in paraffin, sectioned at 4- μ m thickness and stained with hematoxylin and eosin (HE). Colonic epithelial damage was assigned scores as follows: 0 = normal; 1 = hyperproliferation, irregular crypts, and goblet cell loss; 2 = mild to moderate crypt loss (10-50%); 3 = severe crypt loss (50-90%); 4 = complete crypt loss, surface epithelium intact; 5 = small- to medium-sized ulcer (<10 crypt widths); and 6 = large ulcer (\geq 10 crypt widths). Infiltration with inflammatory cells was assigned scores separately for mucosa (0 = normal, 1 = mild, 2 = modest, 3 = severe), submucosa (0 = normal, 1 = mild to modest, 2 = severe), and muscle/serosa (0 = normal, 1 = moderate to severe). Scores for epithelial damage and inflammatory cell infiltration were added, resulting in a total scoring range of 0-12^[9]. All analyses were performed "blind."

Statistical analysis

Data were expressed as mean \pm SE. An unpaired Student's *t* test was used to examine differences in the extent of weight loss, the survival time, and histology scores. This statistical analysis was determined using GraphPad Prism (GraphPad Software, Inc., San Diego, CA). A *P* value less than 0.05 was considered statistically significant.

RESULTS

To test the role of lymphocytes in the development of DSS-induced colitis, we administrated various doses of DSS orally into wild-type control and Rag-1 KO mice and investigated the clinical and pathological findings. We could not detect any differences between B6 control and Rag-1 KO mice when treated with high dose (5%) of DSS. However, the progression of colitis significantly slowed down in Rag-1 KO mice treated with low dose (1.5%) of DSS. To evaluate the disease activity, we observed the symptomatic parameters, including body weight loss, survival rate and diarrhea. The control B6

mice administered with oral DSS lost body weight, almost $20 \pm 3.79\%$ ($n=10$) in 12 d. In contrast, the absence of lymphocyte in Rag-1 KO mice significantly prevented the loss of body weight caused by the treatment of DSS, the decrement being $7.98 \pm 1.79\%$ ($n=14$, $P<0.005$ vs control B6 mice/DSS administration). The differences of body weight began to be statistically significant 8 d after DSS administration and maintained through the experiments (Figure 1A). In addition to weight loss, the survival rates and clinical signs were also different between two groups. At the end of the study, while 65% of control mice died or lost weight more than 20%, only 5.5% (1/18) of Rag-1 KO mice lost weight more than 20% (Figure 1B). Furthermore, the severity of diarrhea after DSS treatment was scored according to the scale described in the Materials and Methods. In control B6 group, DSS caused severe diarrhea, which peaked around day 10, and the average score at day 10 reached the level of 5.545 ± 0.55 ($n=11$). In comparison, the grade of diarrhea was improved significantly in Rag-1 KO mice, with the average score of 1.36 ± 0.54 ($n=14$, $P<0.0001$ vs B6 mice-DSS, Figure 1C). Next, to identify the morphological changes induced by DSS treatment, we examined the changes in the colon. In macroscopic examination, we found that the colons in Rag-1 KO mice treated with DSS were less shortened, although it was not statistically significant (Figure 1D). In addition, macroscopic examination showed fewer intra-abdominal adhesion and less erythematous colons in Rag-1 KO mice (not analyzed statistically).

The severity of colonic inflammation was evaluated further by histopathologic examinations. As shown in Figures 2A-2F, wild-type animals often had massive infiltration of mixed inflammatory cells in mucosa and submucosa with epithelial denudation, disruption of mucosal architecture, ulceration, and muscular thickening. In contrast, inflammatory infiltrates were diminished in the colonic mucosa of Rag-1 KO mice, and many areas appeared intact, implying that the effects of lymphocytes in DSS-induced colitis is detrimental. We scored the histological alterations (histology score, HS) as described in the Materials and Methods^[9] and demonstrated that the differences in histology between two groups were statistically significant (Figure 2G). The HS in wild-type B6 mice treated with DSS (7.80 ± 0.82) was significantly higher than that in Rag-1 KO mice treated with DSS (3.850 ± 0.63 , $P=0.0005$). Taken together, our results suggest that lymphocytes play detrimental roles in the development of DSS-induced colitis.

DISCUSSION

Oral administration of DSS for several days leads to colonic epithelial lesions and acute inflammation characterized by the presence of neutrophils and macrophages within damaged segments. The reason for the deleterious effects of DSS is not well understood, however, epithelial cell toxicity, increased epithelial cell permeability, and macrophage activation have been proposed as potential mechanism^[10]. In addition, it has been controversial whether the development of DSS-induced colitis is affected by the presence of

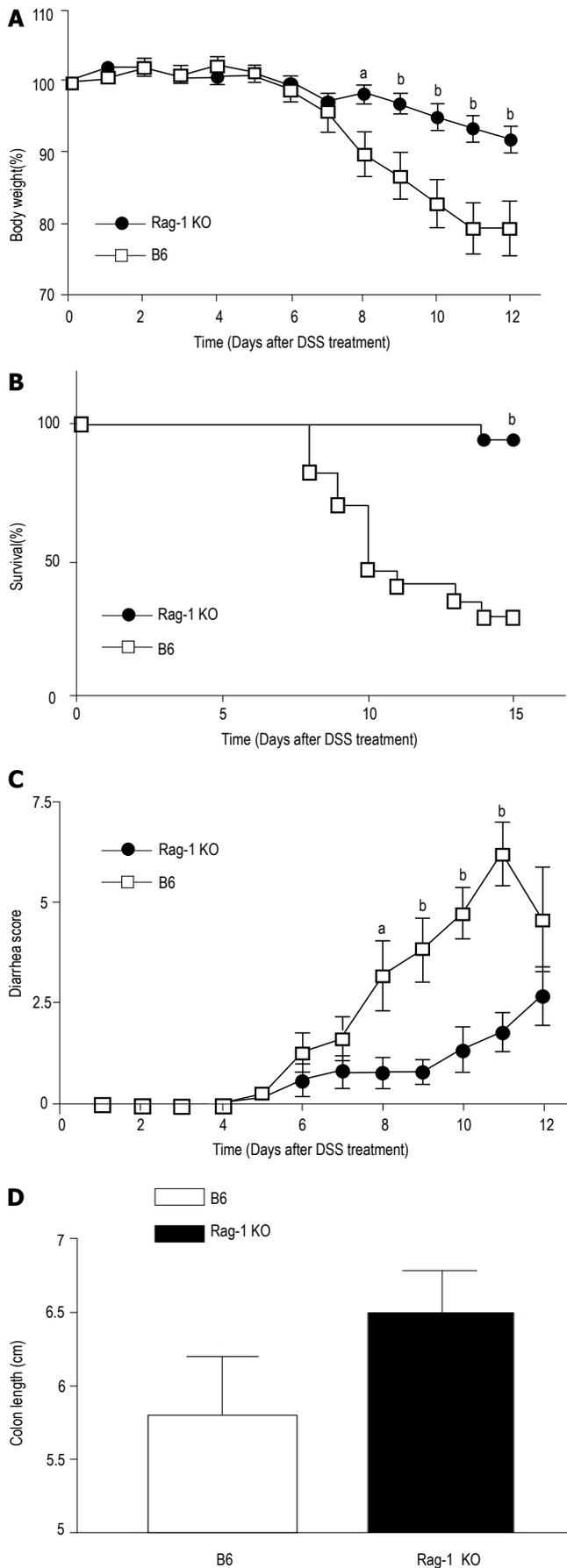


Figure 1 Rag-1 deficient mice showing resistance to develop of DSS-induced colitis. Weight loss (A), mortality (B), the severity of diarrhea (C) and shortage of colon (D) after 1.5% DSS administration were analyzed. Each study group contained > 10 mice. Data shown represent mean values obtained from four independent experiments (^a*P*<0.05; ^b*P*<0.01).

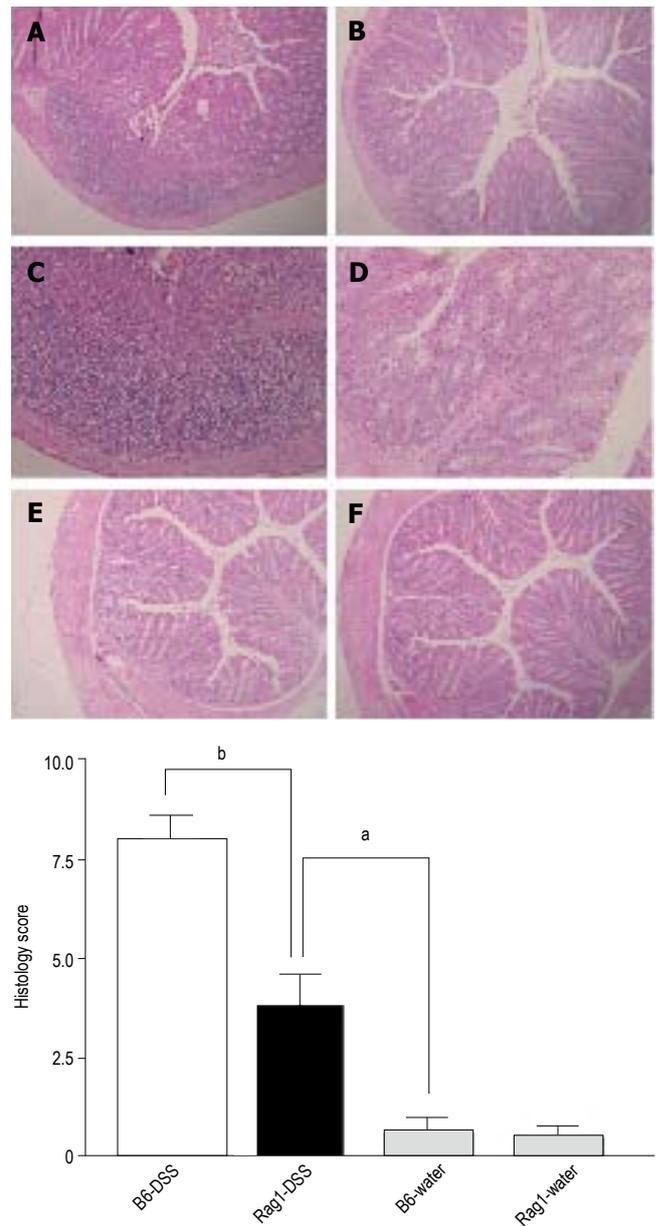


Figure 2 Hematoxylin and eosin (HE) staining of colons obtained from B6 and Rag-1 KO mice treated with or without DSS. A, C: B6 mice treated with DSS (X100 and 160); B, D: Rag-1 KO mice treated with DSS (X100 and 160); E: B6 mice treated with water (X100); F: Rag-1 KO mice treated with water (X100); G: Quantitative histopathologic assessment of DSS-induced colitis activity showing a significant suppression in Rag-1 KO mice compared to the B6 control mice. Samples were collected from B6 control and Rag-1 KO mice treated with 1.5% DSS (open and solid bar, respectively), or water (stripped bars). Data are expressed as mean ± SE and represent > 10 mice per group (^a*P*<0.05; ^b*P*<0.01).

lymphocytes^[1]. To test the role of lymphocyte in the pathogenesis of DSS-induced colitis, we observed the development and clinical course of DSS-induced colitis in both Rag-1 KO and B6 control mice and clearly showed that the absence of lymphocyte suppressed DSS-induced colitis at low dose DSS stimulation

However, while the overall severities were significantly reduced in Rag-1 KO mice, the inflammatory changes and the shortage of the colons were also observed in Rag-1 KO mice compared to that in wild-type B6 control mice. In addition, we could not detect any differences in clinical courses and morphological changes between Rag-1 KO

and B6 control mice when treated with high dose of DSS (5%, data not shown). Taken together, these results suggest that the roles of lymphocytes are dispensable in DSS-induced colitis initiation and considered as an aggravating factor under the presence of weak insults (low dose of DSS)^[1]. Our hypothesis is also supported by a previous paper which showed that adoptive transfer of primed T cells from DSS-treated mice by itself did not induced colitis but aggravated DSS-induced colitis, in contrast to the data from TNBS-treated Th1-type colitis model^[11].

Given the contribution of lymphocyte to DSS-induced pathology, the data presented here lead to the question of “how”. At the moment, this question can only be answered in general terms by the following suggestions: DSS is known to be directly toxic to colonic epithelial cells and disruption of colonic epithelial barriers allows monocytes-macrophages in lamina propria to enter the activation phase^[10] and then macrophages uptake and present foreign peptide into T cells in addition to the secretion of pro-inflammatory cytokines. The activated T cells contribute to DSS-induced pathology as an aggravating factor.

In summary, our results show that lymphocytes play a crucial role in the induction of DSS-induced colitis resembling human ulcerative colitis as aggravating factors.

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RAPID COMMUNICATION

A truncated hepatitis E virus ORF2 protein expressed in tobacco plastids is immunogenic in mice

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Abstract

AIM: To cost-effectively express the 23-ku pE2, the most promising subunit vaccine encoded by the E2 fragment comprising of the 3'-portion of hepatitis E virus (HEV) open reading frame 2 (ORF2) in plastids of tobacco (*Nicotiana tabacum* cv. SR1), to investigate the transgene expression and pE2 accumulation in plastids, and to evaluate the antigenic effect of the plastid-derived pE2 in mice.

METHODS: Plastid-targeting vector pRB94-E2 containing the E2 fragment driven by rice *psbA* promoter was constructed. Upon delivery into tobacco plastids, this construct could initiate homologous recombination in *psaB-trnFM* and *trnG-psbC* fragments in plastid genome, and result in transgene inserted between the two fragments. The pRB94-E2 was delivered with a biolistic particle bombardment method, and the plastid-transformed plants were obtained following the regeneration of the bombarded leaf tissues on a spectinomycin-supplemented medium. Transplastomic status of the regenerated plants was confirmed by PCR and Southern blot analysis, transgene expression was investigated by Northern blot analysis, and accumulation of pE2 was measured by ELISA. Furthermore, protein extracts were used to immunize mice, and the presence of the pE2-reactive antibodies in serum samples of the immunized mice was studied by ELISA.

RESULTS: Transplastomic lines confirmed by PCR and Southern blot analysis could actively transcribe the E2 mRNA. The pE2 polypeptide was accumulated to a level as high as 13.27 µg/g fresh leaves. The pE2 could

stimulate the immunized mice to generate pE2-specific antibodies.

CONCLUSION: HEV-E2 fragment can be inserted into the plastid genome and the recombinant pE2 antigen derived is antigenic in mice. Hence, plastids may be a novel source for cost-effective production of HEV vaccines.

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Key words: Hepatitis E virus; E2; Plastid transformation; Vaccine; Tobacco

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INTRODUCTION

Hepatitis E is a water-borne disease caused by hepatitis E virus (HEV) in many tropical and subtropical areas. Over 50 outbreaks have been reported since its first epidemic in Delhi, India, documented in 1955 and 1956^[1]. It accounts for more than 50% of acute viral hepatitis among young adults in developing countries with a fatality rate of 1-3% in non-pregnant patients and up to 20% in pregnant women^[2]. Consequently, it is a serious threat to public health and has an urgent need of effective vaccines.

HEV appears as a 27-30-nm virus-like particle. It has a ≈ 7.5-kb linear, single-stranded, a positive-sense RNA made up of a short 5'-noncoding region, a 3'-poly(A) tail, and three overlapping open reading frames (ORFs)^[3,4]. ORF1 extends approximately 5 kb from the 5'-end and encodes a polyprotein with functional motifs typical of RNA-dependent RNA polymerase, RNA helicase, papain-like cysteine proteinase and methyltransferase. ORF2 is made up of several dozens of base pairs downstream of the ORF1 and extends approximately 2 kb to the termination codon that is 200-300 bp from the 3'-poly(A) residues. ORF2 contains a consensus signal peptide sequence at its amino terminus and a capsid-like region similar to that seen with other virus capsid proteins. ORF3 partially

overlaps the ORF1 and ORF2, and encodes a 123-aa polypeptide with the possible role of a cytoskeletal anchor site for the assembly of virus particles^[5].

Since killed and attenuated vaccines for hepatitis E are not available due to a lack of culture system for HEV production, recombinant proteins represent the best hope for subunit vaccines. The ORF2-encoded protein has been shown to be the most promising candidate because the only neutralizing epitope identified to date is mapped to its carboxy-terminal region between aa 578-607^[6]. To explore the possibility that these subunit vaccines could be produced through recombinant protein approach, ORF2 fragments have been expressed in prokaryotes^[7], insect cells^[8], animal cells^[9], and transgenic plants^[10]. Satisfactory accumulations of the ORF2 proteins in bacterial^[7] and animal cells^[9] have been achieved. However, if the vaccines are to be produced in such systems, a relatively higher expense may be encountered. In insect cells^[11] and nuclear transformed tomatoes^[10], the ORF2-encoded peptides are accumulated at much lower levels (1-2 mg/L and 48-61 ng/g fresh weight, respectively). Hence, a technique for cost-effective large-scale production of the ORF2 subunit vaccines plays an important role in the control of hepatitis E.

Plastids are unique organelles in plants. Each plant cell accommodates up to 10 000 copies of the plastid genome, indicating the potential of using transplastomic plants to express foreign proteins at elevated levels^[12]. To obtain high-level accumulation of the ORF2-encoded pE2 peptide (aa 394-607), we inserted its corresponding cDNA sequence, the *E2* fragment^[7], into the plastid genomes of tobacco plants (*Nicotiana tabacum* cv. SR1). The *E2* fragment was expressed satisfactorily at RNA and protein levels, and the plastid-produced pE2 was found to be antigenic in mice. Our results suggest that plastid transformation may be an economical alternative for large-scale production of edible HEV vaccines.

MATERIALS AND METHODS

Modification of chloroplast transformation vector pVSR326

A polylinker (upper strand, 5'-CATGGCCGC-GGGGCCCCGCTAGCAGGCCTG CGGCCGCATC-GATGAGCT-3'; lower strand, 5'-CATCGATGCGGCC-GCAGG CCTGCTAGCGGGCCCCCGCGGC-3') was cloned in *NcoI-SacI* site of pVSR326 (GenBank accession no.: AF527485). The obtained pVSR326 derivative was digested by *SacI* and inserted with a (His)₅ fragment (upper strand: 5'-GCGGGGTT CTCATCATCATCATCATG-GTCCGC-3'; lower strand: 5'-GGACCATGATGATGATGATGAGAACCCCGCGC-3'). The resultant plasmid was termed pMLVHisA.

Construction of chloroplast-targeting vector pRB94-E2

A 0.8-kb *E2* fragment originally obtained from a conserved region near the 3'-terminal of the ORF2 in a Chinese HEV strain (GenBank accession no.: D11092) was released by *BamHI-EcoRI* digestion of pGEX-E2^[13] and cloned in the corresponding site in pBluescript KS. Then the 0.8-kb fragment was removed from the resulting plasmid by *SpeI-ClaI* digestion before it was cloned into the *NbeI-ClaI* site in pMLVHisA. This pMLVHisA derivative was digested by *XbaI* and *KpnI* to produce a cassette of

“rice *psbA* promoter-*E2*-rice *psbA* terminator” which was then inserted into the corresponding site in pRB94^[14]. The resulting construct, termed pRB94-E2, has two cassettes: “rice *psbA* promoter-*E2*-rice *psbA* terminator” for pE2 production, and “tobacco *rrn* promoter-*aadA*-tobacco *psbA* terminator” for expressing the spectinomycin-degrading aminoglycoside 3'-adenyltransferase. These two cassettes were flanked by a 1.5-kb *trnG-psbC* arm and a 1.9-kb *psaB-trnM* arm (nt 36 835-38 314 and 38 326-40 205, respectively, in tobacco plastid genome, GenBank accession no.: Z00044) (Figure 1A). These two arms were for homologous recombination upon delivery of pRB94-E2 into chloroplasts.

Plant material

Seeds of tobacco SR1 were germinated on MS medium (Sigma, St. Louis, USA) plus 3% sucrose and 0.6% agar (Sigma). After the seedlings were grown for 3-4 wk, the leaves were placed on petri-dishes with RMOP medium^[15] for one day before being bombarded with a PDS-1000/He Biolistic[®] Particle Delivery System (Bio-Rad).

Chloroplast transformation

DNA for plastid transformation was prepared using the High Purity Plasmid Maxiprep System (Marligen Bioscience Inc., USA). One microgram of pRB94-E2 plasmid was used to coat 1 mg of 0.6- μ mol/L diametric tungsten particles (M13, Bio-Rad) according to the standard procedure. The coated particles were bombarded at 1100 *psi* to tobacco leaves on petri-dishes. One milligram of the coated particle was used for each bombardment. The bombarded leaves were kept on the same petri-dishes for 24 h before being cut into ≈ 1 cm² pieces and put on RMOP plus 500 μ g/mL spectinomycin dihydrochloride. Spectinomycin-resistant shoots emerged in about a month, and their leaves were used as explants for subsequent rounds of regeneration until homoplasmy was achieved.

PCR

Primer pairs (Hel-297/Hel-403, Hel-294/Hel-401 and Hel-354/Hel-355) capable of differentiating WT and transplastomic genomes (Table 1 and Figure 1A) were used. In addition, Hel-19/Hel-24 was used for the amplification of a 1.4-kb fragment in the endogenous *rbhL* gene from both the WT and the transplastomic plants. Thirty-five cycles of PCR were performed using the *Taq* DNA polymerase (Promega). PCR products were separated on a 0.8% agarose gel for visualization under UV illumination after ethidium bromide staining.

Southern blot analysis

Genomic DNA was digested by *NcoI* and *KpnI*, separated on a 0.8% agarose gel and transferred onto a GeneScreen Plus[®] membrane (PerkinElmer), before separate hybridization to four ³²P-labeled DNA probes (P1, P2, P3, and P4 in Figure 2A). Hybridization was performed overnight at 65°C in a buffer containing 250 mmol/L NaCl, 7% SDS and 125 mmol/L phosphate, pH 7.0. After hybridization, blots were washed twice (10 min each) at room temperature in 2 \times SSC plus 0.5% SDS, and once at 65°C for 15min in 0.2 \times SSC plus 0.1% SDS. Blots were then

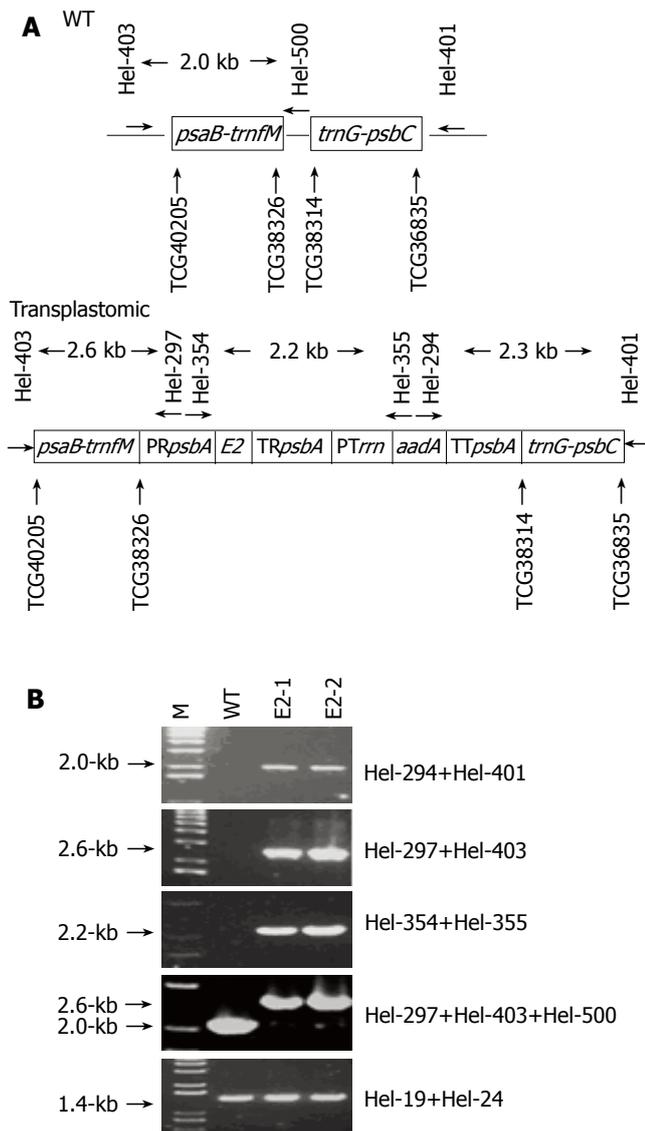


Figure 1 Transplastomy of the spectinomycin-resistant plants. **A:** Maps of WT and transplastomic genomes in the area of two arms (*psaB-trnFM* and *trnG-psbC*) for homologous recombination. Horizontal arrows indicate locations and orientations of different primers. The sizes of PCR products are shown in kb. Arrows pointing upwards denote nucleotide positions in the tobacco chloroplast genome (TCG) in accordance with GenBank accession no. Z00044. *PRpsbA*: rice *psbA* promoter; *TRpsbA*: rice *psbA* terminator; *PTrrn*: tobacco *rrn* promoter; *TTpsbA*: tobacco *psbA* terminator. **B:** Agarose gel pictures of PCR products. Primers used for generating each product are given to the right of the panels. Arrows indicate sizes of the PCR products in kb. Primer pair Hel-19/Hel-24 amplified a 1.4-kb fragment in the *rbcl* gene presenting in both the WT and the transplastomic plants.

exposed to X-ray.

Northern blot analysis

RNA samples were prepared from leaves of WT and transplastomic plants. Seven micrograms of the isolated RNA was separated on 1.2% agarose gel, blotted onto a membrane and hybridized to four ³²P-labeled DNA probes (P4, P5, P6, and P7 in Figure 3A). The *aadA*-specific probes (P4) were the same as those used for Southern blot analysis, while "*psbA-Prn*" (P6) and *trnFM* (P7) probes were made with templates generated by PCR using primer pairs of Hel-720/Hel-721 and Hel-348/Hel-399, respectively (Table 1). Hybridization and washing conditions were the

same as in Southern blot analysis.

Enzyme-linked immunosorbent assay (ELISA)

HEV antigen ELISA Kit and standard pE2 antigen were purchased from Beijing Wantai Biological Pharmacy Enterprise Co., Ltd., Beijing, China. Total soluble protein (TSP) from weighed WT and transplastomic plant tissues (leaves and seeds) was extracted with a non-denaturing buffer [50 mmol/L Tris-HCl (pH 8.5), 10 mmol/L sodium acetate, 100 MgCl₂, 1 mmol/L EDTA and 1 mmol/L PMSF]. After protein quantification with the Bradford agent, 1 μg leaf TSP and 5 μg seed TSP were used for ELISA. Antigen concentrations expressed as nanogram pE2 per microgram TSP were calculated using an ELISA standard curve established with a serial dilution of the standard pE2 antigen. These concentrations were further converted to microgram pE2 per gram fresh tissue using the protein yield (μg TSP/g fresh weight) as multiplication factor. The average values for these factors from three independent assays were 20.95 ± 3.95 and 10.79 ± 1.40 mg TSP/g fresh seeds and leaves, respectively.

Mice immunization with plastid-derived pE2

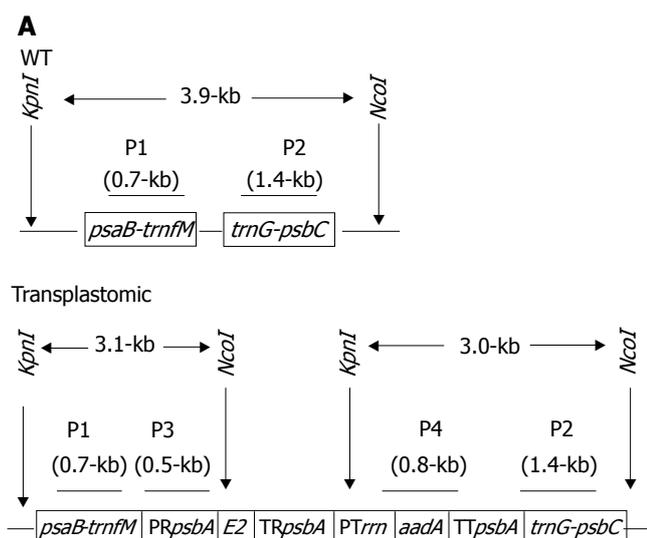
Four-week old BALB/c mice were divided into four groups. Each mouse in the first group was subcutaneously injected with 100 μL of PBS solution, the second group with 100 μg of TSP from WT plant, the third group with 100 μg of TSP from the E2-1 leaves, and the fourth group with 10 μg of TSP (equivalent to ≈ 1 μg pE2) from *E. coli* expressing the pE2 polypeptide. Injection was performed once a week for three consecutive weeks. One week after the last infection, serum samples were collected for pE2-specific antibody detection.

Serum samples were diluted five times with coating buffer (0.015 mol/L sodium carbonate, 0.025 sodium bicarbonate, pH 9.6). Fifty microliters of the diluted samples were added to a 96-well microplate and incubated at 4 °C overnight. After the coating buffer was removed, the wells were blocked with 5% non-fat milk in PBS supplemented with 0.5% Tween 20 for 2 h, which was followed by four washes with 200 μL PBS plus 0.5% Tween 20. Then 200 ng of the pE2 antigen (Beijing Wantai Biological Pharmacy Enterprise Co., Ltd.) diluted in 50 μL PBS plus 0.5% Tween 20 was added and incubated at 37 °C for 1 h. After the uncaptured pE2 was removed and the wells were washed five times, 100 μL of HRP-conjugated monoclonal anti-HEV antibodies (Beijing Wantai Biological Pharmacy Enterprise Co., Ltd.) were added. After incubation at 37 °C for 30 min, the antibody solution was removed and the wells were washed five times. Then 50 μL chromogen A and 50 μL chromogen B were added to the well and the plate was incubated at 37 °C for 2 h. After color development, 50 μL stop solution was added and absorbance at 450 nm (*A*₄₅₀) was measured with equal volume of mixture of chromogen A and chromogen B as reference.

RESULTS

Generation of transplastomic tomato plants

The vector pRB94 was reported to have an increased chloroplast-targeting efficacy^[14]. Hence we used its backbone

**Table 1** Primers for PCR analysis of transplastomic plants

| Name | Sequence (5'-3') | Position |
|---------|--------------------------|---|
| Hel-19 | atgtcaccacaacagag | 57 595-57 612 ¹ |
| Hel-24 | atccaaaacgtccactgc | 59 005-59 002 ¹ |
| Hel-294 | agcccgatcacttgaagctagac | 6 563-6 587 ² , 5 191-5 215 ³ |
| Hel-297 | ataccaatgtcaaccaagccagcc | 2 008-2 032 ³ |
| Hel-348 | gatgatcatagaagcccttacc | 199-222 ² , 38 524-38 547 ¹ |
| Hel-354 | tgcaagcacgatttggggagag | 1 806-1 827 ³ |
| Hel-355 | cagatcaatgtcgcgatctggctg | 6 856-6 878 ² , 4 900-4 922 ³ |
| Hel-399 | tggcaaaaacaagatgttgcggag | 38 341-38 363 ¹ |
| Hel-401 | gttcttaaatccgtgggtggg | 36 683-36 706 ¹ |
| Hel-403 | agaaccaatttcgggattgggcac | 40 312-40 335 ¹ |
| Hel-720 | gtagaagtctagatgcc | 4 300-4 317 ³ |
| Hel-721 | agctttggcgagctagttg | 7 388-7 406 ² |

¹Positions are indicated in accordance with the numbering of the tobacco plastome sequence (accession no. Z00044); ²Positions are indicated in accordance with the numbering of the pRB94 vector (accession no. AJ312392); ³Positions are indicated in accordance with the numbering of pVSR326 vector (accession no. AF527485).

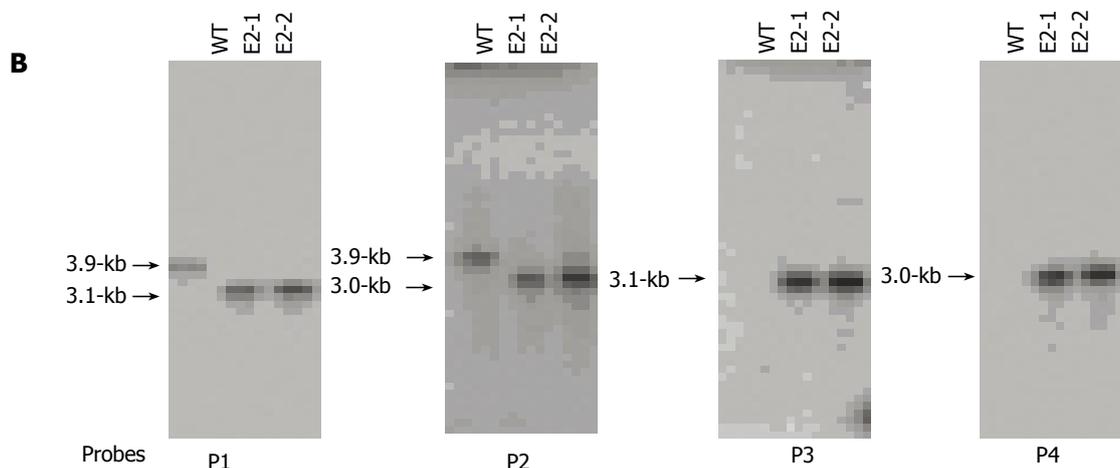


Figure 2 Transformed plastid genomes revealed by Southern blot analysis. **A:** Maps of WT and transplastomic genomes showing the positions of *KpnI* and *NcoI* sites, and areas to which different probes hybridize. The restriction fragment lengths are given in kb. **B:** DNA gel blot analysis confirming transgene integration into chloroplast genomes. DNA was digested by *KpnI* and *NcoI*. The blots were hybridized with probes targeting the *psaB-trnfM* arm (P1), *trnG-psbC* arm (P2), rice *psbA* promoter (P3) and *aadA*-coding region (P4). Arrows indicate the sizes of the hybridized DNA fragments in kb.

and made a construct pRB94-E2 for tobacco chloroplast transformation in this study.

Two shoots were obtained after culturing the bombarded leaf pieces in spectinomycin-containing RMOP medium for about a month. The shoots were selected for two more generations in the same RMOP medium. They were then transferred to grow in greenhouse after root development in a hormone-free MS medium. The resulting plants were named E2-1 and E2-2.

PCR analysis

To confirm whether the spectinomycin-resistant E2-1 and E2-2 were transplastomic, PCR were performed with primer pairs that could only amplify DNA fragments that resulted from homologous recombination (Table 1 and Figure 1A) for which three pairs were used. The first pair (Hel-297/Hel-403) could produce a 2.6-kb product that resulted from recombination in the *psaB-trnfM* arm, the second (Hel-294/Hel-401) a 2.0-kb fragment as a result

of recombination in the *trnG-psbC* arm, and the third (Hel-354/Hel-355) a 2.2-kb fragment from the rice *psbA* promoter to the *aadA*-coding sequence. All the three pairs produced PCR products of expected sizes from E2-1 and E2-2 DNA samples (Figure 1B), indicating that homologous recombination occurred and the *E2*- and *aadA*-expressing cassettes were co-integrated and stayed intact in the plastid genomes.

Southern blot analysis

To confirm the transgene integration and to determine the homoplasmic status, DNA of both the WT and transplastomic plants in the greenhouse was digested by *KpnI* and *NcoI*, and subjected to Southern blot analysis with probes hybridizing to the *psaB-trnfM* arm (P1), *trnG-psbC* arm (P2), rice *psbA* promoter (P3), and *aadA*-coding region (P4). Restriction fragments and their lengths in kb for both the WT and transplastomic genomes are shown in Figure 2A. When probes P1 and P2 were used, the WT

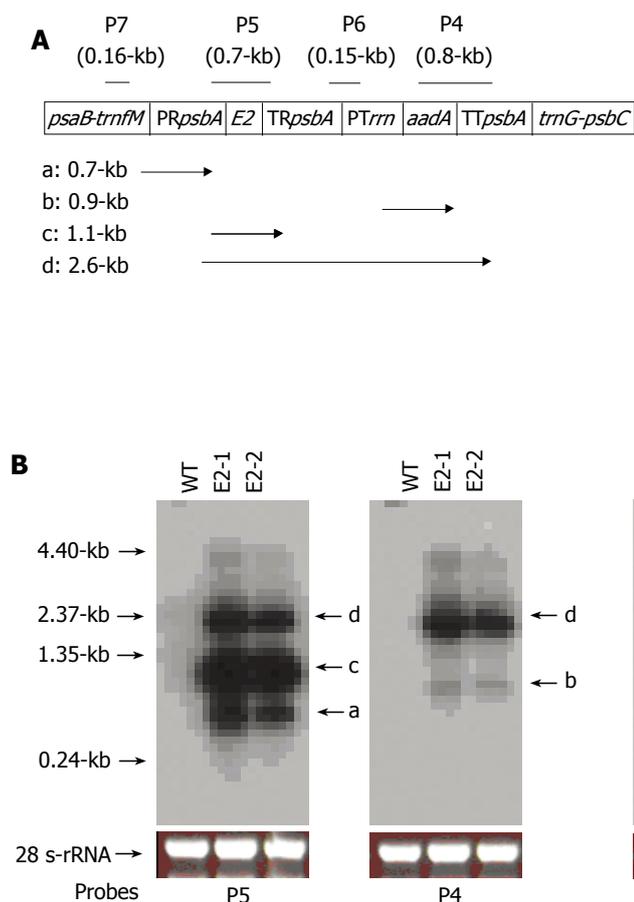


Figure 3 Transgene expression investigated by Northern blot analysis. **A:** Map of the transplastomic genome with areas to which different probes hybridize. Lengths of possible transcripts are shown in kb. **B:** RNA blot analysis for characterization of different transcripts. RNA blots were hybridized with probes targeting the *aadA*-coding region (P4), HEV pE2-coding sequence (P5), junction between the rice *psbA* terminator and tobacco *rrn* promoter (P6) and tobacco *trnFM* (P7). Arrows to the left of the panels denote lengths of RNA in kb. Normalization of RNA loading was demonstrated by ethidium bromide staining of the RNA gel.

revealed a 3.9-kb band, while the transplastomic plants produced bands of 3.1- and 3.0-kb, respectively (Figure 2B). Absence of the 3.9-kb band in the transplastomic plants indicated homoplasmy. When probes P3 and P4 were used, the transplastomic plants showed the expected 3.1- and 3.0-kb bands, respectively (Figure 2B). These results supported the data from PCR (Figure 1) and confirmed that both *E2*- and *aadA*-expressing cassettes were transformed into the chloroplast genome, and the transplastomic plants achieved homoplasmy.

Northern blot analysis

In the E2-1 and E2-2 plants, the transgenes were actively transcribed. When the blot bearing RNAs from both the WT and the transplastomic plants was hybridized with *E2*-specific probes P5 (Figure 3A), three bands (0.7-, 1.1- and 2.6-kb) were detected in the transplastomic plants (bands a, c, and d, Figure 3B), indicating that *E2*-containing transcripts were terminated at multiple locations. When the same blot was hybridized with *aadA* probes P4, a 2.6-kb band and a 0.9-kb band appeared (Figure 3B). The 2.6-kb band represented run-through transcripts initiated from the rice *psbA* promoter, since it could be detected by *E2*-specific probes P5, but not by *trnFM*-targeting probes P7 (Figure 3B). The 0.9-kb band was the *aadA* transcripts plus 0.1-kb 5'-UTR between the *rrn* promoter and *aadA*-coding

Table 2 Accumulation of pE2 leaves and seeds of WT and transplastomic plants (mean \pm SD)

| Plant | Tissue | pE2 | |
|-------|--------|-------------------|---------------------------|
| | | (ng/ μ g TSP) | (μ g/g fresh weight) |
| WT | Leaves | nd | nd |
| | Seeds | nd | nd |
| E2-1 | Leaves | 1.090 \pm 0.182 | 13.273 \pm 2.217 |
| | Seeds | 0.015 \pm 0.004 | 0.261 \pm 0.060 |
| E2-2 | Leaves | 0.630 \pm 0.133 | 5.912 \pm 1.251 |
| | Seeds | 0.018 \pm 0.005 | 0.460 \pm 0.093 |

ND: Non-detectable.

fragment. To determine the initiation sites for the 0.7- and 1.1-kb transcripts, probe P7 was used. In this case, only the 0.7-kb band was revealed (band a, Figure 3B). Therefore, the 0.7-kb transcripts were initiated from *trnFM*, while the 1.1-kb transcripts were solely from the *E2*-expressing cassette, which consisted 0.2-kb *psbA* 5'-UTR, 0.8-kb *E2* and the remaining polylinker sequences (\approx 0.1-kb).

Accumulation of pE2 polypeptide in transplastomic tobacco plants

To investigate if the pE2 antigen was produced in E2-1 and E2-2, TSP extracted from both plants was used in a pE2-specific ELISA assay. An ELISA standard curve was established using different amounts of standard pE2 and used for quantifying the pE2 expressed as amount of fresh pE2 per gram. As high as 13.27 and 0.46 μ g of pE2 were detected in leaves and seeds, respectively (Table 2). In comparison with the concentrations (47.9 ng/g fresh leaf tissue and 61.2 ng/g fresh fruit) of the same pE2 antigen expressed in tomato nuclear transformants^[10], this study achieved over 200-fold increase in leaf pE2 content (Table 2). This elevated level of pE2 accumulation faithfully demonstrated that plastid transformation was superior to nuclear engineering for the production of HEV vaccines.

Antigenicity of plastid-derived pE2 in mice

To investigate if the plastid-derived pE2 proteins were

Table 3 Antigenicity of plastid-derived pE2 in mice

| Group | A450 | |
|--------------------|--------|--------|
| | Expt A | Expt B |
| PBS | 0.117 | 0.137 |
| WT TSP | 0.138 | 0.134 |
| E2-1 TSP | 0.387 | 0.779 |
| <i>E. coli</i> TSP | 1.367 | 2.620 |

antigenic, TSP from leaves of transplastomic plant E2-1 was subcutaneously injected into mice once a week for three consecutive weeks. One week after the last injection, serum samples were collected for antibody detection.

The pE2 polypeptides produced in plastids were antigenic when injected into four-week old BALB/c mice, according to ELISA that specifically detected pE2-reactive antibodies (Table 3). In addition, in consistent with reports from other groups^[7,16], bacterially-expressed pE2 could also stimulate mice to produce the corresponding antibodies (Table 3). Therefore, plastids were an ideal compartment for the production of antigenic pE2 proteins.

DISCUSSION

The unique biological property of plastid-containing plant cells is the high ploidy degree of plastid genomes^[17]. Unlike nuclei, plastids provide an environment where foreign gene activity is not subjected to positional effect and epigenetic silencing. Thus, plastid transformation has the potential to steadily express foreign proteins at elevated level. Compared to the pE2 content in nuclear transformed tomatoes^[10], this study resulted in a dramatic increase in pE2 accumulation and demonstrated that plastid transformation was substantially superior to its nuclear counterpart for pE2 production. High-level pE2 expression in tobacco plastids did not affect growth rates, flowering, seed setting or any other morphological features (data not shown), indicating that no apparent pleiotropic effects occur on the transplastomic plants.

The pE2 can interact with one another to form homodimers that are strongly recognized by serum samples from hepatitis E patients^[13] and highly immunogenic in monkeys^[7] and rats^[16]. These findings reveal two important aspects about the HEV subunit vaccines. The first aspect is that carboxy terminus of the ORF2-encoded major structural protein possesses good antigenicity, and the other aspect is that conformation of these subunit vaccines is crucial in antibody stimulation and in antibody-antigen interaction. Plastid-derived pE2 should therefore possess the right conformation in order to be functional.

Plastids do offer an ideal compartment for foreign proteins to have functional folding. Examples include plastid-expressed nontoxic B subunit of *E. coli* heat-labile enterotoxin^[18] and human somatotropin^[19]. The pE2 expressed in this study reacted with the pE2-specific conformational antibody in the HEV antigen ELISA kit. Such a reaction could be abolished in the presence of SDS and when heating was applied to denature the protein

(data not shown), suggesting that the plastid-derived pE2 possesses functional conformation.

Antibodies cross-reacting with pE2 were detected in serum samples of mice immunized with plastid-derived pE2 in this study. This is consistent with reports that antigenic pE2 can be produced from bacteria^[7,16], and confirms that plastids are a cheaper source for the production of HEV vaccine.

The feasibility of pE2 as a broad-type antigen against HEV has been manifested by several observations. First, with the exception of a Mexican isolate and a USA isolate, the 0.8-kb pE2-coding sequence used in this study shares more than 90% homology with the corresponding sequences in other HEV isolates^[7]. Second, though at least four genotypes have been identified, HEV is antigenically conserved and so far only one serotype has been identified^[1]. Third, all naive macaques infected with HEV genotypes 1, 2, 3, and 4 have been reported to produce antibodies to the ORF2 polypeptides, one of which is comprised of aa 458-607^[20], shorter than the plastid-produced pE2 (aa 394-607) in this study. Hence, we may anticipate that the pE2 peptide in its proper conformation can stimulate antibodies against all the four reported genotypes.

Plastid-encoded genes are typically organized into polycistronic transcription units that give rise to overlapping RNAs. Though the significance of this complicated mode of expression is unknown, the intergenic sequences involved should not possess the typical function of strong terminators. The multiple bands detected with the E2-specific probes (P5) indicate that transcripts initiated from the rice *psbA* promoter could pass through the rice *psbA* terminator. This run-through transcription might actually contribute to the synthesis of the *aadA*-encoded protein, since *aadA*-specific probes revealed much less initiation of the *aadA* transcripts from the tobacco *rrn* promoter. The same *rrn* promoter was able to satisfactorily initiate *aadA* transcription, when the E2 fragment in the pRB94-E2 vector was replaced by the bacterial *uidA* gene (data not shown). Whether proximal DNA sequences influence activity of the *rrn* promoter is unclear at present.

While this study has proved the feasibility of expressing immunogenic pE2 in plastids, the tobacco plant is nevertheless inedible and a transgenic tobacco-based vaccine that can be administered by subcutaneous injection is a feasibility supported by the present work. For hepatitis B, an edible plant-based oral vaccine has been demonstrated in animals^[21]. Introduction of the pE2-coding sequences into the plastid genomes of edible plants such as tomato, lettuce and carrot would thus be more desirable to test if an oral-based vaccine for hepatitis E could be realized. In tomatoes, foreign protein has been shown to express in fruit chromoplasts^[14]. This example provides assurance for the production of edible vaccines through plastid transformation.

In conclusion, transplastomic tobacco plants expressing the antigenic HEV pE2 peptide at elevated level can be successfully generated and it is possible to produce pE2 in edible plant parts for the purpose of immunizing human beings against HEV infection.

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Diagnosis of obscure gastrointestinal bleeding by intra-operative enteroscopy in 81 consecutive patients

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Abstract

AIM: To analyze the results and complications of intra-operative enteroscopy (IOE) by investigating a series of selected patients with bleeding suspected to originate from the small intestine.

METHODS: Eighty-one patients (mean age: 65 years) including 40 males (49.4%) and 41 females (50.6%) with obscure gastrointestinal bleeding underwent IOE between 1990 and 2004. The patients were identified from a database and data were selected from the patients' charts retrospectively. All the patients had undergone at least one non-diagnostic esophagogastroduodenoscopy, colonoscopy, standard enteroscopy and a negative abdominal ultrasound scan before IOE.

RESULTS: The median minimal hemoglobin level in the patients was 59+15g/L and 72.8% of the patients required transfusion of packed erythrocytes previously. A bleeding source was detected in 68 (84%) of the patients during IOE. Angiodysplasias were found in 44 patients (54.3%) and 9 patients (11.1%) were affected by ulcers in the small intestine. A tumor in the small intestine was detected in another 6 patients. The treatment consisted of argon-plasma-coagulation, surgical suture or limited resection in most of the patients.

CONCLUSION: Intra-operative enteroscopy is still used for the diagnosis of suspected small bowel bleeding. Recent developments such as wireless capsule endoscopy and double balloon enteroscopy, may lead to the replacement of IOE in the future.

INTRODUCTION

In most patients suffering from gastrointestinal bleeding, a bleeding source can be detected using upper endoscopy or colonoscopy. However, the small intestine is a source of bleeding in up to 5% of patients^[1,2]. The diagnostic and therapeutic approach to patients with bleeding originating from the small intestine still remains a challenge to all physicians involved in their care.

Several endoscopic and non-endoscopic techniques have been developed for detection of the bleeding sites in the small intestine and their use depends on the severity and time parameters of hemorrhage^[3]. In the last few years, wireless capsule endoscopy^[4,5] and double-balloon enteroscopy^[6,7] have closed the diagnostic gap between colonoscopy and push-enteroscopy for many of the patients. However, intra-operative enteroscopy is still a diagnostic approach that offers the complete endoscopic work-up of the entire small intestine as well as the detection of extramural lesions and the definitive endoscopic or surgical therapy during the same session^[8-11].

The aim of our study was to analyze the results of intra-operative enteroscopy for the diagnosis of gastrointestinal bleeding of obscure origin by investigating a large consecutive series of patients in a third referral center.

MATERIALS AND METHODS

Between 1990 and 2004, 81 consecutive patients with bleeding of obscure origin underwent intra-operative enteroscopy in our department. Patients undergone IOE were identified from a database of the Department of Gastroenterology. The patient charts were analyzed retrospectively with respect to the basic data, such as previously performed examinations, need for transfusion, type of

Table 1 Basic data of the 81 consecutive patients (mean \pm SD)

| Patients (<i>n</i>) | 81 |
|--------------------------|--------------------|
| Age (Yrs) | 65 \pm 20,8 |
| Female (<i>n</i>) | 41 (50.6%) |
| Transfusion need (%) | 72.8 |
| Minimal hemoglobin level | 5.9 \pm 1.5 g/dL |

bleeding (overt or obscure), endoscopic findings through IOE, therapeutic procedures performed and short-term outcome after IOE until discharge from the hospital. Basic patient data are given in Table 1.

All the patients had at least one episode of gastrointestinal tract bleeding with need for transfusion (obscure or overt bleeding), recurrent minor bleeding episodes without transfusion requirement or chronic iron-deficiency-anemia with suspected bleeding of obscure origin. All the patients were subjected to an endoscopic work-up of the colon and the upper gastrointestinal tract before IOE. They all underwent standard enteroscopy (using a sonde-type or push-type enteroscope (Olympus SIF-100 or XSIF-140Q)) and an abdominal ultrasound scan to exclude malformations of the larger abdominal vessels and/or larger tumors in the abdomen. In a large number of patients, additional methods such as barium enema, MR-enteroclysm and angiography or scintigraphy, were applied prior to IOE.

Intra-operative enteroscopy was routinely carried out under general anesthesia. After a median laparotomy, the small intestine was opened through an incision in the middle third. A surgeon placed the endoscope (Olympus PCF-20; PCF-160 HI or CF-Q140) through a sterile sleeve in the intestine. The endoscope was then pushed up to the duodenum and down into the cecum. If a bleeding source was detected, the lesion was treated endoscopically (e.g. argon-plasma-coagulation) or surgically (e.g. resection) during the same session.

Data were entered with a standard program (Microsoft Excel) and analyzed by the statistical module. Data were expressed as median and range if not stated elsewhere. The study was conducted in accordance with the Declaration of Helsinki.

RESULTS

The median minimal hemoglobin level before IOE was 59 \pm 15 g/L and 72.8% of the patients received at least two units (0-50) of packed erythrocytes.

The patients had a median of 4 EGDs and underwent colonoscopies before the IOE procedure. All the patients were subjected to at least one push-type enteroscopy and/or abdominal ultrasound, 80% of them had a non-diagnostic small bowel enema (Figure 1). During the push-enteroscopy prior to IOE, single small angiodysplasias were found in 5 cases within reach of the enteroscope and treated with argon plasma coagulation. IOE found additional angiodysplasias. One additional patient had a non-bleeding small ulcer in the upper jejunum and was exam-

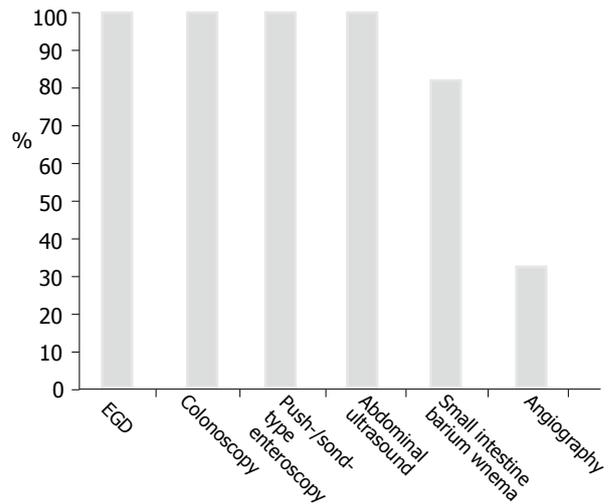


Figure 1 Proportion of patients undergone different endoscopic procedures before intra-operative endoscopy.

ined intraoperatively because of ongoing hemorrhage.

Intra-operative enteroscopy was able to visualize the complete small intestine in all the patients. A bleeding source was detected in 68 (84%) of the patients. Angiodysplasia was found in 44 (54.3%) patients and ulcer in 9 patients (11.1%). The ulcer might be due to the use of non-steroidal anti-rheumatics (NSAR) and the presence of Crohn's disease. Using IOE, we found tumors of the small intestine not identified previously in 6 patients (7.4%) of the study group. Four of the tumors were malignant, namely 1 B-cell lymphoma, 2 neuroendocrine tumors, 1 gastrointestinal stromal tumor. Two additional tumors were benign, namely 1 Peutz-Jeghers polyp and 1 giant hyperplastic polyp. Four patients of the series had a Meckel's diverticulum, two of them having a Meckel's scintigraphy prior IOE without any signs of a Meckel's diverticulum. Three patients had large diverticula of the jejunum or ileum, which were presumed to be the site of bleeding. Additional diagnoses included Dieulafoy's ulcer in the cecum (missed by the previously performed colonoscopy) and bleeding from anastomotic vessels after cholecystojejunostomy. Intra-operative enteroscopy was non-diagnostic in 13 patients (16%) (Figure 2).

All patients with a positive finding during IOE were treated. Angiodysplasias were treated with argon-plasma-coagulation (APC) ($n=20$), with a combination of APC and surgical suture in case of large malformations ($n=18$), or with surgical resection of a small part of the small intestine ($n=6$) in case of multiple angiodysplasias located in well-defined areas of the ileum. Both the tumors and all the large diverticula were completely resected in all the 6 patients during the IOE session.

One of the patients, an 84-year-old woman with recurrent blood loss originating from the small intestine and a minimal hemoglobin level of 43 g/L, had an intra-abdominal abscess due to a leakage at the incision site of the small intestine. She was re-operated two days after the IOE and recovered soon without further problems. There was no mortality due to the intra-operative enteroscopy or its sequelae during her hospital stay (Figure 3).

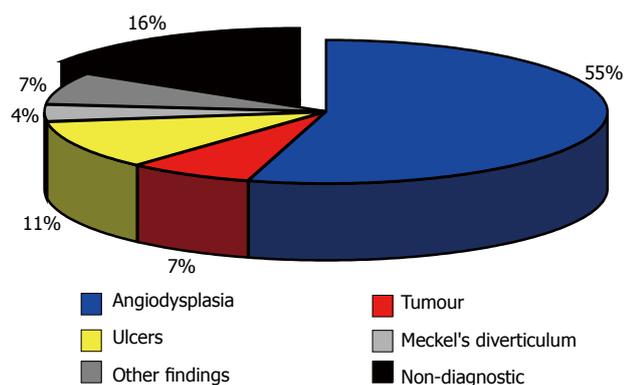


Figure 2 Diagram of the main diagnoses acquired during IOE. Angiodysplasia was the most frequent finding. Other findings were one Dieulafoy ulcer in the cecum (not-diagnosed during a previous colonoscopy). Three patients had a Meckel's diverticulum and 3 patients had a large diverticulum in the ileum ($n=1$) or jejunum ($n=2$).

DISCUSSION

The management of patients with obscure gastrointestinal bleeding remains a diagnostic and therapeutic challenge to all physicians involved in their care^[3]. After non-diagnostic upper and lower endoscopy, the use of peroral push-type enteroscopy has been the diagnostic standard procedure for many years^[12,13].

Push enteroscopy is still the method of choice for suspected small intestinal bleeding as it is easy to perform and is available in several endoscopic centers. Push-enteroscopy provides the option for a broad spectrum of diagnostic and therapeutic endoscopic procedures, including biopsies, argon-plasma-coagulation, injection methods and even removal of polyps^[13]. However, this technique is limited with respect to the insertion depth due to the tortuous nature of the small intestine and looping in the stomach. In most cases, only the upper and middle part of the jejunum can be reached and the diagnostic harvest is thus limited. In several series for which push-enteroscopy was used for the diagnosis of a bleeding source, the rate of the diagnostic findings ranges 15-53%, depending on the timing of endoscopy, the type of enteroscope and the clinical setting^[5,13-15].

For many years, IOE has been the only method for evaluating the mucosal surface of the complete small intestine. This procedure is limited to patients who are operative candidates and have recurrent or severe bleeding episodes^[16], since IOE is an invasive procedure requiring general anesthesia. Several methods for IOE such as peroral approach, transanal and direct intubation through a surgical incision of the small intestine are now available. The latter is method used in our series and allowed for the examination of the entire small intestine in all the patients. In general, the diagnostic yield of IOE is high when patients are highly selected for such procedures. It was reported that IOE can detect 70-80% sources of bleeding^[17]. In our series, a probable or definite bleeding source was found in 68 (84%) of the 81 patients. The IOE procedure allowed for the endoscopic or surgical treatment of the lesions during the same session in all our patients.



Figure 3 Typical angiodysplasia in the ileum diagnosed during IOE.

The most common finding by IOE was angiodysplasia with a rate of 54.3%. In several other series investigated for small intestinal bleeding, angiodysplasias are the most common findings too and local treatment results in the reduction of blood loss in a substantial portion of patients^[15,18,19].

Four of the 81 patients had previously non-diagnosed malignant tumors of the small intestine and 6 additional patients were affected by a Meckel's diverticulum or a large small intestinal diverticulum as a bleeding source. These conditions required operative treatment rather than endoscopic treatment even if they were within the reach of the scope. In our series, we encountered only one major complication during hospital stay (intra-abdominal abscess formation). Most published series present a comparable low morbidity rate and no mortality^[8-11,20]. Though the accuracy and diagnostic yield of IOE are high the procedure should be regarded as the last resort for patients with unexplained obscure gastrointestinal bleeding due to its invasive nature^[21].

Wireless capsule endoscopy and double-balloon enteroscopy constitute new methods for the diagnosis of patients with obscure gastrointestinal bleeding. Capsule endoscopy allows for non-invasive visualization of the entire small intestine in a substantial portion of patients. It was reported that it has a high diagnostic yield of angiodysplasia, ulcer and active bleeding in selected populations of obscure gastrointestinal bleeding^[5]. A recently published Italian multi-center study on capsule endoscopy examined 100 patients with obscure gastrointestinal bleeding and yielded 47% positive and 15% suspicious findings^[22]. The timing of capsule endoscopy is important: in patients with ongoing overt bleeding during the examination, the findings are positive in 92%. When capsule endoscopy was performed more than 10 d after an overt bleeding episode, the diagnostic rate decreased to 12%. However, in 21% of cases, the cecum was not reached, and in 5% of the cases, asymptomatic retention of the capsule occurred. Capsule endoscopy is limited in its use because biopsies and interventional treatment procedures are not possible and the localization of findings is sometimes difficult. A two-center-study showed that the sensitivity of capsule endoscopy (compared by IOE as a criterion) is 95%, the positive and negative predictive values are 95% and 86%, respectively^[23].

Double balloon enteroscopy (DBE) is a new diagnostic option that has the potential to replace intra-operative enteroscopy in the near future^[7]. It allows for endoscopic visualization of the entire small intestine in a substantial portion of patients. The technical principle of DBE is an alternating push and pull procedure^[6].

Initial data from Japan and Germany show encouraging results, with a high diagnostic yield of DBE in patients with suspected small bowel diseases. However, in only 10-15% patients, visualization of the complete small intestine is possible. Two procedures, beginning perorally and then transanally, are required for a substantial portion of patients. The procedure is time-consuming. In patients having undergone previous surgical operations of the abdomen, a complete examination of the small intestine is not be possible because of adhesions. As this method is carried out without general anesthesia and its nature is less invasive compared to IOE, repeated DBE is possible even in older patients and in those with recurrent bleeding.

Studies comparing IOE with DBE have not yet been conducted. Such studies may never be performed because patients with a diagnostic DBE do not need an IOE and vice versa. Nevertheless, further studies should be carried out to redefine the role of the invasive IOE procedure in the new era of wireless capsule endoscopy and double-balloon enteroscopy.

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Expression of Interleukin-11 and Interleukin-11 receptor α in human colorectal adenocarcinoma; Immunohistochemical analyses and correlation with clinicopathological factors

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Abstract

AIM: There is strong evidence that interleukin-11 (IL-11) is involved in the regulation of tumor progression, cellular growth and differentiation. Recently, interleukin-11 receptor (IL-11R) has been detected on some cancer cells. In this study, we investigated the expression of IL-11 and IL-11R in colorectal adenocarcinoma.

METHODS: To elucidate the involvement of IL-11 and IL-11R α in human intestinal adenocarcinomas, we examined 115 cases of surgically resected human colonic adenocarcinoma and 11 cases of adenoma by immunohistochemistry and Western blotting.

RESULTS: Among 115 cases of adenocarcinoma, 100 cases (87.0%) showed positive staining in the cytoplasm of carcinoma cells for the IL-11, and 87 cases (75.6%) were positive for the IL-11R α . Six cases (54.5%) and four cases (36.4%) of 11 adenomas were positive for IL-11 and IL-11R α , respectively. The expression of IL-11R α correlated with the histological differentiation ($P=0.033503$), the depth of tumor invasion ($P=0.006395$), Dukes' classification ($P=0.015648$) and lymphatic invasion ($P=0.003865$). However, the expression of IL-11R α was not correlated with the venous invasion and the presence of lymph node metastasis. The expression

of IL-11 was not correlated with any clinicopathological factors. In Western blot analysis, two human colorectal carcinoma cell lines and four tissues of surgically resected human carcinoma expressed both IL-11 and IL-11R α proteins.

CONCLUSION: IL-11 and IL-11R α are highly expressed in human colorectal adenocarcinoma and the IL-11R α expression is correlated with clinicopathological factors. These findings suggest that the expression of IL-11R α is an important factor for the invasion of human colorectal adenocarcinoma.

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Key words: IL-11; IL-11 receptor α ; Colorectal cancer

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INTRODUCTION

Colorectal cancer is one of the most common neoplasms and is a significant cause of morbidity and mortality^[1,2]. Consequently, colorectal carcinoma is one of the best characterized examples of multistep progression in which activation of oncogenes, such as the *K-ras* gene^[3], and inactivation of tumor suppressor genes, such as APC, DCC and p53^[4-7] take place. The prognosis of colorectal cancer patients is based on the depth of tumor cell invasion and the presence of lymph node metastasis. Recently, it has been suggested that the occurrence and progression of cancer is related to the activation of some cytokines and/or an intracellular signaling pathway. But the development and progressive mechanism of the colorectal cancer is not fully understood.

IL-11 was cloned from the primate stromal cell line PU-34 and was initially considered to be a hematopoietic

Table 1 Expression of IL-11 and IL-11R α in colorectal carcinoma, (%)

| | <i>n</i> | IL-11 | | | IL-11R α | | |
|------------------------------|----------|--|-----------|-----------|--|-----------|-----------|
| | | - | + | ++ | - | + | ++ |
| Normal mucosa | | ¹ <i>P</i> =0.001883, <i>vs</i> carcinoma | | | ¹ <i>P</i> =0.00196, <i>vs</i> carcinoma | | |
| | 10 | 5 (50.0) | 3 (30.0) | 2 (20.0) | 6 (60.0) | 4 (40.0) | 0 (0.0) |
| Adenoma | | ¹ <i>P</i> =0.023291, <i>vs</i> carcinoma | | | ¹ <i>P</i> =0.000833, <i>vs</i> carcinoma | | |
| | 11 | 5 (45.5) | 2 (18.2) | 4 (36.4) | 7 (63.6) | 4 (36.4) | 0 (0.0) |
| Total carcinoma | 115 | 15 (13.0) | 26 (22.6) | 74 (64.3) | 28 (24.3) | 31 (27.0) | 56 (48.7) |
| Histological differentiation | | NS | | | ¹ <i>P</i> =0.033503 | | |
| Well | 57 | 8 (14.0) | 15 (26.3) | 34 (59.6) | 17 (29.8) | 16 (28.1) | 24 (42.1) |
| Moderate | 46 | 5 (10.9) | 8 (17.4) | 33 (71.7) | 6 (13.0) | 14 (30.4) | 26 (56.5) |
| Poorly | 5 | 0 (0.0) | 1 (20.0) | 4 (80.0) | 1 (20.0) | 0 (0.0) | 4 (80.0) |
| Mucinous | 7 | 2 (28.6) | 2 (28.6) | 3 (42.9) | 4 (57.1) | 1 (14.3) | 2 (28.6) |
| Depth of tumor invasions | | NS | | | ¹ <i>P</i> =0.006395 | | |
| Tis | 24 | 4 (16.7) | 6 (25.0) | 14 (58.3) | 8 (33.3) | 10 (41.7) | 6 (25.0) |
| T1 | 11 | 1 (9.1) | 5 (45.5) | 5 (45.5) | 6 (54.5) | 2 (18.2) | 3 (27.3) |
| T2 | 10 | 1 (10.0) | 1 (10.0) | 8 (80.0) | 2 (20.0) | 3 (30.0) | 5 (50.0) |
| T3 | 48 | 7 (14.6) | 9 (18.8) | 32 (66.7) | 8 (16.7) | 10 (20.8) | 30 (62.5) |
| T4 | 22 | 2 (9.1) | 5 (22.7) | 15 (68.2) | 4 (18.2) | 6 (27.3) | 12 (54.5) |
| Dukes' classification | | NS | | | ¹ <i>P</i> =0.015648 | | |
| A | 44 | 6 (13.6) | 13 (29.5) | 25 (56.8) | 16 (36.4) | 15 (34.1) | 13 (29.5) |
| B | 35 | 4 (11.4) | 6 (17.1) | 25 (71.4) | 4 (11.4) | 8 (22.9) | 23 (65.7) |
| C | 32 | 4 (12.5) | 6 (18.8) | 22 (68.8) | 7 (21.9) | 7 (21.9) | 18 (56.3) |
| D | 4 | 1 (25.0) | 1 (25.0) | 2 (50.0) | 1 (25.0) | 1 (25.0) | 2 (50.0) |
| Lymphatic invasion | | NS | | | ¹ <i>P</i> =0.003865 | | |
| Present | 76 | 11 (14.5) | 14 (18.4) | 51 (67.1) | 15 (19.7) | 16 (21.1) | 45 (59.2) |
| Absent | 39 | 4 (10.3) | 12 (30.8) | 23 (59.0) | 13 (33.3) | 15 (38.5) | 11 (28.2) |
| Venous invasion | | NS | | | NS | | |
| Present | 44 | 5 (11.4) | 10 (22.7) | 29 (65.9) | 8 (18.2) | 11 (25.0) | 25 (56.8) |
| Absent | 71 | 10 (14.1) | 16 (22.5) | 45 (63.4) | 20 (28.2) | 20 (28.2) | 31 (43.7) |
| Lymphnode metastasis | | NS | | | NS | | |
| Present | 35 | 5 (14.3) | 6 (17.1) | 24 (68.6) | 8 (22.9) | 7 (20.0) | 20 (57.1) |
| Absent | 80 | 10 (12.5) | 20 (25.0) | 50 (62.5) | 20 (25.0) | 24 (30.0) | 36 (45.0) |

NS: Not significant, ¹statistical analyses are performed by Spearman's correlation coefficient by rank test or Mann-Whitney's *U* test methods.

cytokine^[8,9]. It was found later to have effects on non-hematopoietic systems, and to act on many different cells and tissues, which included stimulation of megakaryocyte maturation, platelet production, and growth stimulation of CD34+ hematopoietic progenitor cells^[8,9]. This cytokine has also been shown to mediate inhibition of adipogenesis^[10] and stimulation of osteoclasts^[11], and acts as a cytoprotective agent of gut mucosa during treatment with radiation and chemotherapy and in models of inflammatory bowel disease^[12-15].

The IL-11 receptor (IL-11R) is a member of the gp130-dependent receptor group, which includes the receptors for interleukin-6 (IL-6), leukemia inhibitory factor (LIF), ciliary neurotrophic factor and oncostatin M, and it has been associated with various unique biological actions^[16]. IL-11-induced signaling is mediated by the formation of a receptor complex, composed of two molecules each of IL-11, two subunits of the IL-11R α , and gp130. An important signaling system activated by the IL-11R α and other members of this receptor family is the PI3

kinase pathway, MAP kinase pathway and the Janus kinase signaling transducer and activator of transcription (Jak-STAT) pathway^[17].

Recent studies have shown that IL-11R expression was not only present in megakaryocytes, osteoclasts and colon epithelium but also in breast, ovarian and prostate cancers^[18-20]. This suggested that it was theoretically possible that IL-11 could affect the growth of tumor cells. In contrast, one study showed that IL-11 may be involved in the normal growth control in the intestinal epithelium, which suggests that the inhibition by IL-11 is lost during carcinogenic transformation^[21]. In addition, another study showed that IL-11 appears unlikely to stimulate the growth of the most common solid tumors^[22]. Since, IL-11 could become a therapeutically important compound for providing supportive care for patients who receive cancer chemotherapy, it is important to establish that IL-11 does not stimulate growth of tumor cells.

In this study, we investigated the expression of IL-11 and IL-11R α in human colorectal adenocarcinoma and compared that with the clinicopathological factors to elucidate the relationship of IL-11 and IL-11R in colorectal carcinoma.

MATERIALS AND METHODS

Human colorectal tissues and cell lines

Human colorectal tissues were obtained from fresh surgical samples or paraffin-embedded blocks as described previously. All specimens were obtained from patients operated at Nagasaki University Hospital between 1998 and 2003. We examined 115 cases of human colorectal adenocarcinoma and 11 cases of adenoma as benign lesions with moderate dysplasia. Each tumor was assigned a histological type according to the Japanese Classification of Colorectal Carcinoma by the World Health Organization classification^[23] and a depth grading of infiltration according to the TNM staging system by the American Joint Commission on Cancer^[24]. 24 mucosal carcinomas (Tis), 11 submucosal infiltrative carcinomas (T1), 10 carcinomas invading proprial muscle layers (T2), 48 carcinomas reaching the subserosa (T3), and 22 carcinomas penetrating the serosal surface (T4) were examined. Histologically, 57 were well-differentiated adenocarcinomas (wel), 46 were moderately-differentiated adenocarcinomas (mod), 5 were poorly differentiated adenocarcinomas (por), and 7 were mucinous carcinomas (muc) (Table 1).

Colo320DM (adenocarcinoma) and DLD-1 (adenocarcinoma) cell lines derived from human colorectal cancer were obtained from the Human Health Resources Bank (Osaka, Japan). All cell lines were incubated at 37 °C in a humidified incubator containing 5% CO₂ and 95% air. Colo320DM was maintained in RPMI 1640 (Invitrogen Corp., Carlsbad, CA, USA) supplemented with heat-inactivated 10% fetal calf serum (Invitrogen Corp.). DLD-1 was maintained in DMEM/F-12 (Invitrogen Corp.) supplemented with 10% fetal calf serum.

Immunohistochemistry

Formalin-fixed and paraffin-embedded tissues were cut

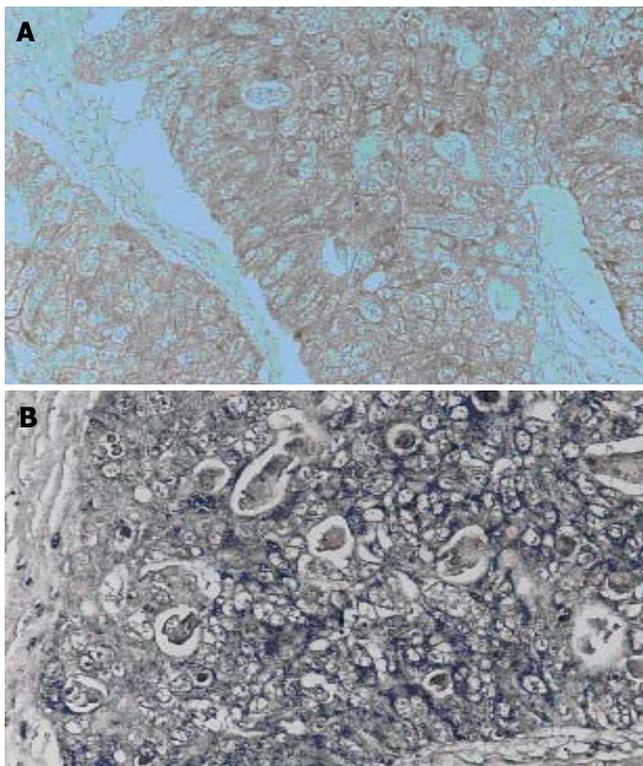


Figure 1 Immunohistochemical staining for the IL-11 and IL-11R α in human colorectal carcinoma. (A) is for IL-11, and (B) is for IL-11R α . IL-11 and IL-11R α show strong cytoplasmic and membranous expression.

into 4 μ m sections, deparaffinized in xylene and rehydrated in phosphate-buffered saline. Deparaffinized sections were preincubated with normal bovine serum to prevent nonspecific binding, and then incubated overnight at 4°C with an optimal dilution (0.1 μ g/mL) of a primary polyclonal rabbit antibody against human IL-11 (H-169) and IL-11R α (N-20). Each antibody was bought from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The slides for IL-11 were then sequentially incubated with a horseradish-conjugated goat antirabbit immunoglobulin G antibody. And the reaction products were resolved using a diaminobenzidine (DAB; DAKO, Carpinteria, CA, USA). The slides for IL-11R α were sequentially incubated with an alkaline phosphatase-conjugated goat antirabbit immunoglobulin G antibody, and the reaction products were resolved using a mixture of 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium chloride (BCIP/NBT; DAKO). Primary antibodies preabsorbed with excess recombinant IL-11 and IL-11R α peptides (Santa Cruz Biotechnology, Inc.), respectively, were used as negative controls. Prostatic cancer tissues^[19] served as the internal positive control for IL-11 and IL-11R α immunostaining. Analysis of the immunohistochemical staining was performed independently by two investigators (K. Yamazumi and T. Nakayama). IL-11 and IL-11R α expression was classified into three categories depending on the percentage of cells stained: -, 0 to 10% positive cells; +, 10 to 50% positive tumor cells; and ++, > 50% positive tumor cells.

Western blot analysis

Western blot analysis for IL-11 and IL-11R α expression was

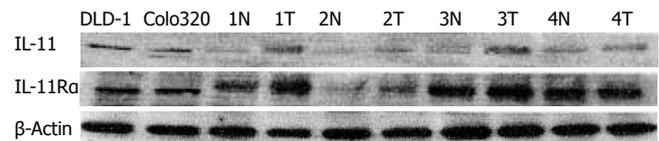


Figure 2 Demonstration of IL-11 and IL-11R α in colorectal carcinoma cells and tissues by Western blotting. All of colorectal carcinoma cells, carcinoma tissues and normal mucosa expressed both IL-11 and IL-11R α . (N: normal mucosa, T: human colorectal cancer tissue).

performed on four human colorectal carcinoma tissues, normal mucosal tissues and two colorectal carcinoma cell lines. Human colorectal tissues were obtained within 1 hour of surgery and immediately frozen. The tissues were then suspended in RIPA buffer (50 mmol/L Tris, 150 mmol/L NaCl, 1% NP-40, 1% sodium deoxycholate and 0.05% SDS, pH 7.4), broken into pieces on ice and subjected to three freeze-thaw cycles. The insoluble cell debris was removed by centrifugation at 14000 r/min at 0°C for 10 min. The supernatant was collected and the protein concentration was quantified using a protein assay reagent (Bio-Rad Laboratories, Hercules, CA). The proteins (20 μ g) were separated by polyacrylamide gel electrophoresis (PAGE) under denaturing and reducing conditions, and then transferred to a Hybond ECL nitrocellulose membrane (Amersham Biosciences, Buckinghamshire, UK). The membranes were rinsed in TBS, blocked with 5% low-fat dried milk in TBS containing 0.1% Tween 20 (TBS-T), and then incubated for 1 h at room temperature with a 1 μ g/mL dilution of the anti-human IL-11 or IL-11 receptor α antibody (Santa Cruz Biotechnology, Inc.). After extensive washing of the membranes with TBS-T, they were incubated for 1 h with a 1:1000 dilution of the horseradish-peroxidase-conjugated donkey anti-rabbit immunoglobulin G (Santa Cruz Biotechnology, Inc.) in TBS-T containing 3% low-fat dried milk. The membranes were washed and developed with a horseradish peroxidase chemiluminescence detection reagent (ECL Plus System, Amersham Bioscience), and then exposed to Hyperfilm ECL (Amersham Bioscience).

Statistical analysis

The Stat View II program (Abacus Concepts, Inc., Berkeley, CA) was used for statistical analyses. Analyses comparing the degrees of IL-11 or IL-11R α expression were performed by Spearman's correlation coefficient by rank test or Mann-Whitney's *U* test methods.

RESULTS

IL-11 was localized in the cytoplasm of the carcinoma cells (Figure 1A), and IL-11R α was localized both in the membrane and the cytoplasm, mainly in the membrane (Figure 1B). No staining with anti-IL-11 or anti-IL-11R α was seen in the negative controls for tissue staining. Although normal epithelium and stromal tissue around tumors expressed IL-11 and IL-11R α , the expression in these tissues were more faint than that in the carcinoma cells.

Immunohistochemical results are summarized in the Table 1. Among 115 cases of adenocarcinoma, 100 cases

(87.0%) showed positive staining for the IL-11, and 87 cases (75.6%) were positive for the IL-11R α . Among 10 cases of normal colorectal mucosa, 5 cases (50.0%) expressed IL-11 and 4 cases (40.0%) were positive for IL-11R α . And there were significant differences between IL-11 and IL-11R α expression and in between normal mucosa and adenocarcinoma ($P=0.001883$, $P=0.00196$, respectively). Among 11 cases of adenoma, 6 cases (54.5%) were expressed IL-11 and 4 cases (57.1%) of adenoma were positive for IL-11R α , and there were significant differences of IL-11 or IL-11R α expressions between adenoma and adenocarcinoma ($P=0.023291$, $P=0.000833$, respectively).

IL-11 and IL-11R α were expressed more intensely in the invasive than the superficial parts of the carcinoma. The expression of IL-11R α correlated significantly with histological differentiation ($P=0.000833$), depth of tumor invasion ($P=0.006395$), Dukes' classification ($P=0.015648$) and lymphatic invasion ($P=0.003865$) (Table 1). However, there was no correlation with lymph node metastasis and venous invasion. On the other hand, there was no correlation between IL-11 expression and clinicopathological factors.

IL-11 and IL-11R α expression was also detected in two colorectal carcinoma cell lines and all of four human carcinoma tissues by Western blot analysis (Figure 2). The IL-11 protein of 19.1 Kd was clearly detected in all of the two cell lines and four carcinoma tissues. The IL-11R α expression was detected in the two cell lines and four carcinoma tissues at various levels. IL-11 and IL-11R α proteins were also expressed in normal mucosa but were more intense in the cancer tissue than in the normal mucosa (Figure 2).

DISCUSSION

IL-11 is well known as a hematopoietic cytokine. However, the expression of IL-11 has also been observed in many types of cells, such as fibroblasts, chondrocytes, synovial epithelium, bronchial epithelium and colonic epithelium^[15,25,26], and it has been shown that IL-11 has various actions on hepatic, stromal, epithelial, neural and osteoclastic cells^[27]. Recent studies have shown that IL-11R was not only expressed in megakaryocytes, osteoclasts and colon epithelium but also in breast cancer, ovarian cancer and prostate cancer^[12-14]. In this study, we have demonstrated for the first time that IL-11 and IL-11R α are expressed in the human colorectal mucosa, colorectal cancer tissue and cell lines. Both IL-11 and IL-11R α were expressed simultaneously in the carcinoma cell. Therefore, it is suggested that the tumor cell may respond to the IL-11 in an autocrine and/or paracrine fashion to promote the invasion of tumors.

There were statistically significant correlations between the expression of IL-11R α and histological differentiation, tumor invasion and lymphatic invasion. These results suggest that IL-11R α is involved in the regulation of cellular differentiation and tumor progression in human colorectal carcinoma. Recently, the expression of IL-11R α has been reported in carcinoma of the breast, ovary and prostate^[12-14]. We suggest that IL-11 might play an important role in the progression of carcinomas.

IL-11 is known as the activator of the JAK/STAT pathway through GP130^[11]. A recent study showed that STAT3 signaling directly regulates tumor invasion and metastasis^[28]. And other GP130-dependent cytokines, such as IL-6 and LIF, have been reported to have connections with several tumors^[29-33]. Especially, the tumor-promoting and invasion activity of IL-6 has been well studied^[30-32]. IL-11 also shares the GP130 connection, and in one report it was noted that a complex of the soluble IL-11 receptor and IL-11 act as an IL-6-type cytokine^[34]. Thus, it is suggested that IL-11 may stimulate colorectal carcinoma cell growth and invasion through activation of the GP130/STAT-3 signaling pathway.

Recently, it was suggested that IL-11 could become a therapeutically important molecule in supportive care of cancer patients who receive chemotherapy^[35], and another study showed that IL-11 appears unlikely to stimulate the growth of the most common solid tumors^[16]. However, in this study, we have demonstrated that IL-11 may up-regulate the activity of colorectal carcinoma cell growth and/or invasion, so it is necessary to pay very careful attention to the therapeutic use of IL-11.

In conclusion, we have demonstrated that IL-11R α is an important factor involved in the invasion of human colorectal adenocarcinoma. However, the roles of IL-11 and IL-11R in human colorectal carcinoma remain unclear and await further study.

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RAPID COMMUNICATION

Inhibitory effect of angiotensin II receptor antagonist on hepatic stellate cell activation in non-alcoholic steatohepatitis

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Abstract

AIM: To investigate the efficacy of angiotensin II receptor antagonist on hepatic stellate cells (HSCs) activation in the patients with non-alcoholic steatohepatitis (NASH).

METHODS: Seven patients with NASH were prescribed losartan, a selective angiotensin II type 1 receptor antagonist (50 mg/d) for 48 wk. Liver biopsies were performed both at the entry and end of the study in all patients. Quiescent and activated HSCs were identified by double immunostaining using anti-p75 and α -smooth muscle actin antibodies, and the number of each phenotype was counted. Similarly, the liver specimens obtained from the eight patients with non-alcoholic fatty liver (NAFL) were also examined as controls.

RESULTS: In NASH hepatic tissues, activated HSCs were dominantly distributed as compared with those in NAFL. The 48-wk losartan treatment induced a remarkable decrease in activated HSCs and a mild increase in quiescent phenotypes.

CONCLUSION: Our data suggest the crucial involvement of HSCs in anti-fibrotic effect of angiotensin II receptor antagonist on patients with NASH.

INTRODUCTION

Non-alcoholic steatohepatitis (NASH) is a distinct clinical entity characterized by steatosis, varying degrees of lobular inflammation and fibrosis in the liver, and this disease can potentially progress^[1,2]. Previous data showed that about 20% of the patients developed to liver cirrhosis^[3] and several cases of hepatocellular carcinoma are related to NASH^[4]. Although the causes of NASH are not well defined, hepatic stellate cell (HSC) is suggested to play a pivotal role in the progression of hepatic fibrosis as other chronic liver diseases^[5,6]. Stimulated HSCs transform into myofibroblast-like cell, and produce a large amount of extracellular matrix components, resulting in the formation of fibrosis^[7]. However, to our knowledge, the efficacy of any treatments for NASH on the activation of HSCs has not been reported yet. Recently, we have demonstrated that losartan, a selective angiotensin II type 1 receptor antagonist, improved hepatic necroinflammation and fibrosis in patients with NASH^[8]. Based on our results, we hypothesized that an angiotensin II receptor antagonist inhibits the progression of hepatic fibrosis through the inactivation of HSCs in NASH. In this study, we, therefore, investigated the efficacy of long-term losartan treatment on HSCs in patients with NASH.

MATERIALS AND METHODS

Patients

Seven patients with both NASH and hypertension, which was defined as a systolic blood pressure over 140 mmHg

Table 1 Characteristics and biochemical data of patients with non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH) before losartan treatment

| Parameters | NAFL (n=8) | NASH (n=7) |
|--------------------------|------------------|----------------------------|
| Age (yr) Male/female | 59 (34-76) 4/4 | 59 (41-65) 2/5 |
| BMI (kg/m ²) | 24.5 (21.4-31.4) | 26.1 (22.6-36.7) |
| SBP (mmHg) | 130 (92-156) | 148 (127-171) |
| DBP (mmHg) | 78 (54-96) | 90 (71-100) |
| AST (IU/L) | 54 (42-70) | 84 (20-128) |
| ALT (IU/L) | 72 (56-110) | 132 (54-138) |
| γ-GTP (IU/L) | 30 (17-43) | 53 (42-127) ^a |
| TC (mg/dL) | 209 (169-254) | 222 (160-319) |
| TG (mg/dL) | 128 (86-198) | 124 (65-295) |
| FBS (mg/dL) | 95 (86-112) | 109 (97-132) ^a |
| Ferritin (ng/mL) | 111 (30-264) | 199 (54-379) |
| HA (ng/mL) | 46.5 (10.0-78.3) | 89.4 (12.3-197.0) |
| IV collagen (ng/mL) | 2.7 (2.0-3.5) | 5.2 (3.1-9.9) ^a |

Values are expressed as median (range). ^a*P*<0.05 vs NAFL; BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; AST: aspartate aminotransferase; ALT: alanine aminotransferase; γ-GTP: γ-glutamyl transpeptidase; TC: total cholesterol; TG: triglyceride; FBS: fasting blood glucose; HA: hyaluronic acid; IV collagen: type IV collagen 7S.

and/or a diastolic blood pressure over 90 mmHg, and eight patients with non-alcoholic fatty liver (NAFL) were enrolled into this study. A detailed alcohol history was taken by at least two physicians and confirmed by at least one close relative who lived with the patient. All patients consumed less than 40 g of alcohol per week, and were negative for hepatitis B serological tests, antibody to hepatitis C virus or any autoantibodies, including anti-nuclear antibody, and anti-mitochondrial antibody. Serum ceruloplasmin and α1-antitrypsin levels were normal in all patients. Four patients in NASH group and three in NAFL group were obese (body mass index >25); four in NASH and one in NAFL had diabetes mellitus; two in NASH and four in NAFL had impaired glucose tolerance; three in NASH and four in NAFL had hyperlipidemia; and one patient in each group had hyperuricemia. Four of eight patients with NAFL group had hypertension.

Study design

At the entry of the study, some of the patients had already been on medication, including benzodiazepines, calcium antagonists or anticoagulants for at least 12 mo. These therapeutic regimens were not changed after an induction of losartan treatment in patients with NASH. No patient was taking any angiotensin converting enzyme inhibitors or angiotensin II receptor antagonists before the study. Laboratory examination showed abnormally high serum transaminase and γ-glutamyl transpeptidase concentrations in all patients. A liver biopsy was performed prior to entry the study, which revealed a moderate to severe lobular steatosis in both groups. On the other hand, various degrees of hepatic necroinflammation and fibrosis were demonstrated only in NASH group. After the estimation at the entry, patients with NASH were prescribed losartan at a dose of 50 mg/d for 48 wk, and a physical and laboratory examination were monitored every 4 wk. A liver biopsy was repeated at the end of the study in the patients with

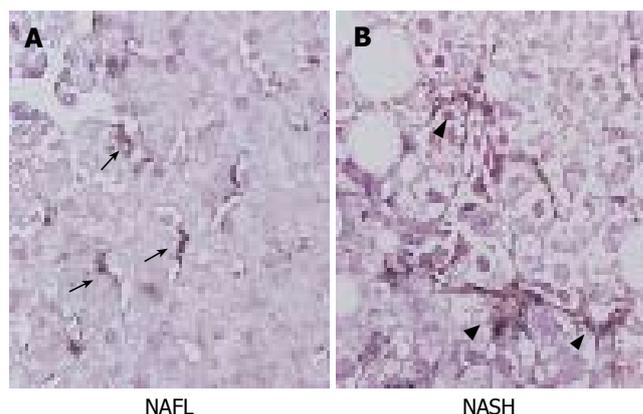


Figure 1 Expression of HSCs in patients with NASH and NAFL (×400). Liver sections were immunostained with both monoclonal anti-p75 antibody and monoclonal anti-α-SMA antibodies. Although most perisinusoidal HSCs were quiescent form (arrow heads) in NAFL, a number of arrow head were detected in the patient with NASH (arrow heads).

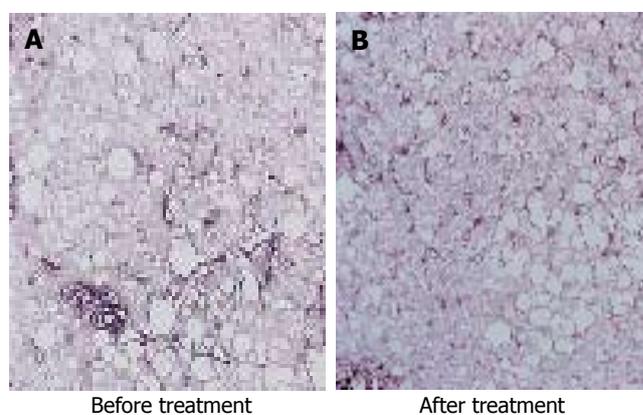


Figure 2 Significant decrease of activated and quiescent HSCs by 48-wk losartan treatment in a patient with NASH (double immunostaining with anti-p75 and α-SMA antibodies, ×200).

NASH. The Ethical Committee of Asahikawa Medical College for Clinical Study approved all protocols, and informed consents were obtained from all patients.

Liver histology

Hematoxylin & eosin and Azan stainings were performed on fixed histological sections. Two experienced hepatopathologists graded necroinflammation and staged fibrosis in a blinded fashion according to the Grading and Staging system of NASH proposed by the American Association for the Study of Liver Diseases^[9]. To identify HSCs, double immunostaining was performed using monoclonal anti-p75 (a low affinity nerve growth factor receptor) antibody (Novocastra, Newcastle, UK) and monoclonal anti-α-smooth muscle actin (α-SMA) antibody (Dako, Kidlington, UK) as previously described^[10,11]. Color development was achieved with BCIP/NBT for p75 and DAB for α-SMA, and counter-staining was performed using direct fast red. Activated HSCs were distinguished from quiescent HSCs by their myofibroblast-like form and the disappearance of fat vesicles in cytoplasm^[7]. The numbers of both activated and quiescent HSCs were blindly counted by two independent pathologists and averaged in five fields per slide at

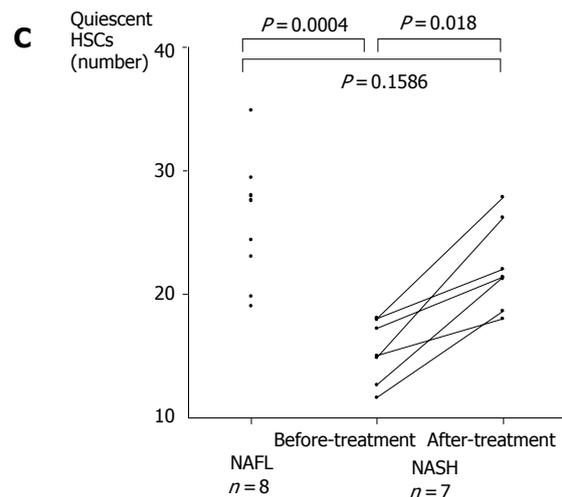
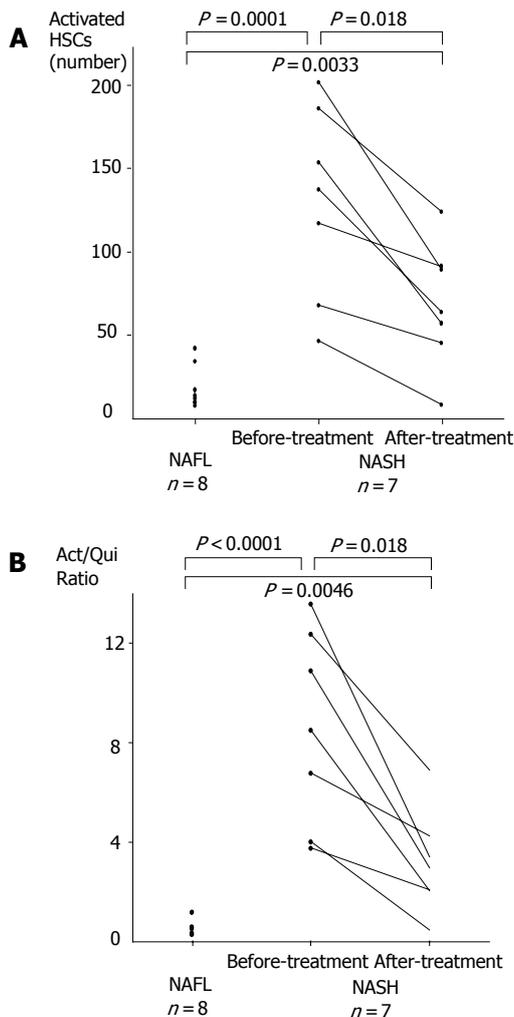


Figure 3 Average numbers of HSCs in five fields per slide at x200 magnification. **A:** number of activated HSCs; **B:** averaged ratio of activated HSCs to quiescent HSCs (Act-/Qui- Ratio: a ratio of activated HSCs to quiescent HSCs); **C:** averaged number of quiescent HSCs.

and circular cell bodies containing lipid droplets in the cytoplasm, were dominantly distributed, and active forms of HSC were rarely observed. On the other hand, activated HSCs (myofibroblast-like phenotype), characterized by enlarged cell bodies, fewer lipid droplets and the presence of membranous processes, were conspicuously scattered in NASH liver tissues. These activated HSCs were preferentially associated with areas of fibrosis (Figure 2). The number of activated HSCs and ratio of activated/quiescent HSCs in NASH liver tissues were significantly higher as compared with those in NAFL liver tissues (Figures 3A and 3B). On the other hand, the number of quiescent HSCs was significantly lower in NASH liver tissues as compared with those in NAFL liver tissues (Figure 3C). In patients with NASH, the number of activated HSCs in the liver sections with mild necroinflammation (grade 1) was significantly lower as compared with those with moderate to severe necroinflammation (grades 2 and 3) [grade 1: 57 (46-68); grades 2 and 3: 153 (117-201), $P < 0.05$]. Although the number of activated HSCs in the liver sections with mild fibrosis (stages 1 and 2) tended to be lower as compared with those with severe fibrosis (stages 3 and 4), there was no statistically significant difference [stages 1 and 2: 68 (46-185); stages 3 and 4: 145 (117-201)].

The number of activated HSCs and the ratio of activated/quiescent HSCs were significantly decreased after 48-wk losartan treatment (Figures 3A and 3B). On the other hand, quiescent HSCs slightly increased after losartan treatment (Figure 3C). There was no significant correlation between the decrease rate of activated HSCs and the improvement of hepatic pathologic findings after losartan treatment.

DISCUSSION

In the present study, we demonstrated that the activated HSCs and the ratio of activated/quiescent HSCs in NASH hepatic tissues were significantly higher as compared with those in NAFL, and activated HSCs in NASH livers were

× 200 magnification.

Statistical analysis

Data were expressed as median values with ranges. Mann-Whitney *U*-test was used to compare two independent data, and Wilcoxon rank sum test was used for paired data. A *P* value < 0.05 was considered statistically significant.

RESULTS

Before the study, serum γ -glutamyl transpeptidase, type IV collagen 7 S and fasting blood glucose levels in NASH group were significantly higher as compared with NAFL group. Although body mass index, serum transaminase, hyaluronic acid, ferritin and triglyceride levels in the NASH group tended to be higher as compare with the NAFL group, there was no statistically significant difference (Table 1). No side effect was noted, and the body mass index was unchanged during the losartan treatment in patients with NASH. The necroinflammatory grade and stage of fibrosis diagnosed by liver biopsies were improved after losartan treatment [for grade: pretreatment, 2 (1-3), posttreatment, 1 (1-2), $P < 0.05$; for stage: pretreatment, 3 (1-4), posttreatment, 2 (1-4), $P < 0.05$].

HSCs were identified in the perisinusoidal area of both NAFL and NASH liver sections (Figure 1). In NAFL liver tissues, quiescent forms of HSC, characterized by small

preferentially localized along fibrotic area. The number of activated HSCs tended to be correlated with the necroinflammatory grade and fibrotic stage. This result was very consistent with previous studies that demonstrated the activation of HSCs in NASH^[5,6], and suggested that the hepatic necroinflammation activated and proliferated HSCs, and led to progress the hepatic fibrosis in NASH. In contrast, Wasington *et al*^[6] reported no difference in the number of activated HSCs between NASH and NAFL. This discrepancy may be due to the difference of employed procedure for identifying activated HSCs. Although we judged activated HSCs by their distinctive form and disappearance of fat vesicle, together with double staining with anti-p75 and α -SMA antibodies, they identified activated HSCs only by positive staining for α -SMA. Since recent studies demonstrated that both activated and quiescent HSCs expressed α -SMA^[10,11], activated HSCs identified by their procedure may include quiescent phenotypes.

We also investigated the changes of HSCs after 48-wk losartan treatment in patients with NASH. Both the number of activated HSCs and the ratio of activated/quiescent HSCs were significantly decreased, and quiescent HSCs were slightly increased by losartan treatment concurrently with the improvement of hepatic fibrosis. The depletion of activated HSCs might be due to inactivation of activated HSCs^[12], or disappearance of activated HSCs by apoptosis^[10]. An increase of quiescent HSCs after losartan treatment supported the former mechanism. However, an increase in quiescent HSCs was relatively small as compared with a decrease in activated HSCs after losartan treatment, suggesting that the latter mechanism may be dominant in a depletion of activated HSCs. Because it is difficult to recruit patients with both NASH and hypertension, and only seven patients were evaluated in this study, significant correlation between the decrease rate of activated HSCs and the improvement of hepatic pathologic findings after losartan treatment was not observed. However, our present data strongly suggested a causative role of HSCs in hepatic fibrosis of NASH.

Although detailed mechanisms for the suppressive effect of angiotensin II receptor antagonist on HSC activation could not be well defined in this study, we hypothesize that there are two different pathways. The first may be the direct inhibition to HSCs. Studies have revealed that an activated HSC expresses angiotensin II type 1 receptor^[13], and synthesizes angiotensin II by itself^[14]. Locally generated angiotensin II enhances the activation and proliferation of HSCs through autocrine and paracrine mechanisms^[15]. Losartan inhibited activated HSCs possibly through the blocking of angiotensin II type 1 receptors expressed on the surface of HSCs. In accordance with this mechanism, angiotensin II receptor antagonist may have a therapeutic efficacy on other chronic liver diseases that HSCs are involved in the progression of hepatic fibrosis, including viral hepatitis, alcoholic liver injury, or autoimmune liver diseases^[16-18]. Actually, Terui *et al*^[19] reported that losartan improved the hepatic fibrosis in chronic type C hepatitis.

Another mechanism of the angiotensin II receptor antagonist may be the indirect inhibition of HSCs mediated through the improvement of hepatic necroinflammation. Chronic hepatic inflammation enhances the production of

transforming growth factor- β 1 (TGF- β 1) from Kupffer cell and inflammatory cells^[20]. TGF- β 1 is a multifunctional and ubiquitous cytokine, and in chronic liver diseases, this peptide plays a causative role in hepatic fibrogenesis through the activation of HSCs. We demonstrated that the patients with NASH had elevated plasma TGF- β 1 levels that were reversed by α -tocopherol^[21]. Moreover, we have recently reported that losartan improves hepatic necroinflammation concurrently with a reduction of plasma TGF- β 1 levels in the patients with NASH^[8]. Angiotensin II is known to aggravate several pathogenic factors of NASH, such as insulin resistance, oxidative stress and hepatic iron overload^[22-24]. In fact, our previous studies demonstrated that serum ferritin levels were significantly decreased and hepatic iron deposition disappeared in some patients with NASH after losartan treatment^[8]. Angiotensin II receptor antagonist may also suppress the HSC activation through the decrease of TGF- β 1.

In conclusion, inhibiting the action of angiotensin II by losartan results in a remarkable depletion of activated HSCs and a mild increase of quiescent HSCs, thereby suggesting the important involvement of HSCs in anti-fibrotic effect of angiotensin II receptor antagonist on the patients with NASH.

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Clinical analysis of propofol deep sedation for 1,104 patients undergoing gastrointestinal endoscopic procedures: A three year prospective study

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Abstract

AIM: To analyze the hemodynamic and respiratory effects of propofol on patients undergoing gastroscopy and colonoscopy.

METHODS: In this prospective study, conducted over a period of three years, 1,104 patients referred for a same day GI endoscopy procedure were analyzed. All patients were given a propofol bolus (0.5-1.5 mg/kg). Arterial blood pressure (BP) was monitored at 3 min intervals and heart rate and oxygen saturation (SpO₂) were recorded continuously by pulse oximetry. Analyzed data acquisition was carried out before, during, and after the procedure.

RESULTS: A statistically significant reduction in mean arterial pressure was demonstrated ($P < 0.001$) when compared to pre-intervention values, but severe hypotension, defined as a systolic blood pressure below 60 mmHg, was noted in only 5 patients (0.5%). Oxygen saturation decreased from 96.5% to 94.4 % ($P < 0.001$). A critical decrease in oxygen saturation ($< 90\%$) was documented in 27 patients (2.4%).

CONCLUSION: Our results showed that propofol provided good sedation with excellent pain control, a short recovery time and no significant hemodynamic side effects if carefully titrated. All the patients (and especially ASA III group) require monitoring and care of an anes-

INTRODUCTION

Gastrointestinal endoscopy remains to date an essential diagnostic and therapeutic tool. Patient comfort during the procedure is of paramount importance for successful completion of the examination^[1,2]. A significant subset of patients is unable to tolerate gastrointestinal endoscopic procedures without sedation^[3,4]. Midazolam and benzodiazepines are most commonly used, often in combination with pethidine, whereas anesthetic agents are less frequently used because oversedation may induce respiratory depression, hypotension, and other cardiopulmonary complications.^[5-8] Optimal administration of conscious sedation and patient monitoring during endoscopy has not been adequately emphasized so far^[9].

The optimal strategy of conscious sedation should be tailored to the individual patient, based on the experience of the gastroenterologist and anesthesiologist. Oversedation may induce respiratory depression and delayed recovery in elderly patients and in those with inherent cardiopulmonary compromise. Hypoxemia and hypotension represent the majority of complications observed, especially in upper intestinal endoscopy^[11], and may occur more frequently during endoscopic procedures than during anesthesia.

Gastrointestinal procedures require careful patient monitoring especially in the high-risk patient population. Patient vital signs have been monitored in less than 25.9% of cases, in the published literature^[9].

Table 1 ASA physical status classification

| ASA group | Procedure | | | Total |
|-----------|-------------|-------------|-----------------|-------|
| | Colonoscopy | Gastroscopy | Both procedures | |
| I | 335 | 209 | 165 | 709 |
| II | 172 | 90 | 98 | 360 |
| III | 14 | 11 | 10 | 35 |
| Total | 521 | 310 | 273 | 1104 |

ASA-American Society of Anesthesiology.

The purpose of this study was to analyze the hemodynamic and respiratory effects of propofol on patients undergoing gastroscopy and colonoscopy and thus determine whether the monitoring and care of an anesthesiologist is required.

MATERIALS AND METHODS

Patients

We analyzed 1104 patients (639 women and 465 men) admitted for a same day colonoscopy (521 patients), gastroscopy (310 patients) or both procedures (273 patients). The study was conducted prospectively over a three year period, from the 1st January 2001 to the 1st January 2004, at the Bates Clinic in Zagreb, Croatia. The median age of our patients was 53 years (range 17-88). Age, sex, body weight, blood pressure, heart rate, electrocardiogram, oxygen saturation, as well as patient history including current medication were recorded. We used the American Society of Anesthesiology classification system (ASA grades I-IV) to stratify patients by risk prior to the gastrointestinal procedure. Seven hundred and nine patients were in ASA group I (healthy patients), 361 in ASA II (patients with disease of one body system), and 35 in ASA III (patients with disease of more than one body system) (Table 1). After written informed consent had been obtained, an intravenous cannula was inserted. All patients were monitored throughout the procedure by the anesthesiologist.

Procedure

The patients were given an intravenous propofol (2,6-diisopropylphenol, Diprivan, Astra Zeneca, USA) bolus (0.5-1.5 mg/kg). The required dose was calculated by the anesthesiologist based on the patient's weight, age, physical condition, and estimated duration of procedure. A mean dose of 135 mg (60-480 mg/kg) of propofol was administered. After an initial dose of 0.5-1.5 mg/kg (ASA I and II) or 0.25-0.5 mg/kg in patients ASA class III or over 70 years, the additional bolus injection was administered to maintain the sedation if needed. Supplemental nasal oxygen was administered at 4 l/min during the procedure. Oxygen saturation and heart rate were monitored continuously by pulse oximetry and blood pressure was recorded at three minute intervals. These values were obtained before, during and after the endoscopic procedure (Table 2). Following the completion of the procedure, the patients were transferred to a recovery room and were closely observed for 30 min. The anesthesiologist recorded an overall pain score (using graded questionnaire describing pain

Table 2 Data on basic laboratory parameters measured before, during and after GI procedures

| Parameter | Findings in patients (mean [±] /SD) | | | Number of monitored patients | | |
|----------------------|--|-------------------------------|-------------------------------|------------------------------|------------------|-----------------|
| | Before procedure | During procedure | After procedure | Before procedure | During procedure | After procedure |
| SBP (mmHg) | 140.8 [±] / -26.0 | 112.2 [±] / -25.3 | 114.6 [±] / -23.7 | 1 096 | 880 | 1 037 |
| DBP (mmHg) | 80.6 [±] / -13.3 | 68.4 [±] / -14.1 | 70.1 [±] / -12.9 | 1 096 | 880 | 1 035 |
| HR (beats/min) | 88.4 [±] / -19.1 | 81.3 [±] / -14.3 | 80.1 [±] / -13.9 | 1 012 | 888 | 1 049 |
| SpO ₂ (%) | 96.5 [±] / -2.9 | 94.4 [±] / -4.1 | 95.3 [±] / -3.4 | 997 | 885 | 1 046 |

Abbreviations: SBP-systolic blood pressure, DBP-diastolic blood pressure, HR-heart rate, SpO₂-oxygen saturation, SD-standard deviation.

as: no pain, mild, moderate and severe pain), complications and recovery time.

Statistical analysis

Contingency tables were made for qualitative data and distribution parameters (mean, standard deviation, minimum and maximum) were calculated for all measured variables (systolic and diastolic blood pressure, heart rate, oxygen saturation and propofol per kg body weight). Paired t-test was used to test differences between pairs of values for all measured hemodynamic variables before, during and after the procedure.

RESULTS

We analyzed arterial blood pressure, oxygen saturation and heart rate (Table 2). Blood pressure and heart rate decreased during the procedure ($P < 0.0001$) and increased after ($P < 0.0001$) an initial value. Our results showed that propofol in dosages of 0.5-1.5 mg/kg decreased the systolic blood pressure from 149.8 to 112.2 mmHg, diastolic blood pressure from 80.6 to 68.4 mmHg and heart rate from 88.4 to 81.3 beats/min. Hypotension, defined as a blood pressure below 60 mmHg, was recorded in 5 patients and they received a 500 mL normal saline bolus. Bradycardia, defined as a heart rate less than 50/min, was recorded in 7 patients (0.6%) and they received 0.5 mg of atropine. All medications were administered by the attending anesthesiologist. Oxygen saturation also decreased during the procedure from 96.5% to 94.4% ($P < 0.001$). Oxygen saturation of less than 90% was documented in 27 patients (2.4%). Seven of them were in ASA class III with cardiopulmonary disease, 14 patients with hypertension and obesity and 6 patients were older than 80 years. All hypoxemic episodes occurred in patients undergoing an upper GI examination. No episodes of apnea occurred and mechanical ventilation was not employed in any of our patients. The hypoxemia proved to be transient in all the patients. In our study 3 patients developed ventricular premature beats whereas 5 patients went into a supraventricular tachycardia with a ventricular rate exceeding 140 beats/min. The endoscopic procedures themselves caused no complications. Total

colonoscopy was achieved in all but 6 patients who had subtotal stenosis. None of our patients reported any pain. Median recovery time was 7 min (range 5-15). Five patients had nausea but no intervention was needed. There were no serious respiratory or hemodynamic complications.

DISCUSSION

Our choice of agent for the establishment of conscious sedation was propofol, a short acting anesthetic agent. In comparison with conventional sedation using midazolam or benzodiazepines, it provides a considerably more rapid onset of action and shorter recovery time^[10,19,20]. We believe it is a safe alternative for patients undergoing endoscopic procedures. In this study, none of the patients sedated with propofol reported any pain, and the mean recovery time was 7 min (5-15 min).

The choice of sedative in GI procedures is largely operator dependent, but generally consists of benzodiazepines used either alone or in combination with an opiate^[5-8]. Such combination may increase the risk of oxygen desaturation and cardiorespiratory complications^[2,4,9]. Trojan *et al*^[14] demonstrated that the residual effects of midazolam on psychomotor function could be documented for at least 1 h after its administration. Paradoxical reactions, including hyperactive or aggressive behavior have been reported^[4]. The anesthetic agents, such as droperidol, propofol and general anesthesia are reserved for patients who remain uncooperative on standard regimens or who are perceived to be at high risk for agitation unless a deeper level of sedation is achieved^[15-17]. General anesthesia is used most commonly in children. Sedation with midazolam, benzodiazepines, analgetics and propofol was administered in many studies by the nurse and the endoscopist^[10,14,18-20]. In certain settings, assistance from an anesthesiologist may be required. Some authors suggest that GI procedures without sedation are satisfactory^[4,6,9,18], but in our previous study^[21] we showed that 50% of patients without sedation reported the procedure as painful.

Our results showed that propofol in dosages of 0.5-1.5 mg/kg decreased the systolic and diastolic blood pressure and heart rate during the procedure and increased after an initial value. Hypotension and respiratory depression represent the majority of the complications observed^[10,11,22-24]. In our study only 5 patients had hypotension and 7 patients developed bradycardia. Most of these patients were obese with cardiopulmonary disease and a compromised general physical condition (ASA class III). Electrocardiographic changes during GI procedures, especially gastroscopy, are common and reported in patients with known heart disease as well as otherwise healthy patients^[24]. Approximately a half of all the complications observed during gastroscopy are of cardiopulmonary origin^[11]. These rhythm abnormalities in 7 patients were of short duration and caused no hemodynamic compromise.

Monitoring of cardiopulmonary function during endoscopic procedures is of outmost importance and we believe that a significant reduction in morbidity and mortality can thus be achieved. The most widely used

definition of hypoxemia is an oxygen saturation of below 90% and monitoring of oxygen saturation is more sensitive than a clinical detection of cyanosis. Respiratory complications with oxygen desaturation were recorded in 2.4% patients in our study. We prevented hypoxemia with administration of supplementary of 4l/min oxygen. Numerous studies have documented the occurrence of hypoxemia during endoscopy^[6,12,23-24]. They reported cardiopulmonary complications with oxygen desaturations in 40-60% of patients with sedation, and some studies have reported desaturation in 40% of unsedated patients^[23]. Obesity, pulmonary disease, age and mechanical airway obstruction worsened hypoxemia. Their recommendation consisted of pulse oximetry monitoring. Intermittent oxygen desaturation is also common during sleep in normal subject^[25]. Others showed that hypoxemia can be prevented by providing supplemental oxygen^[5,23]. Gastrointestinal societies in the United States and United Kingdom issued guidelines for monitoring and oxygen administration^[23,24]. All patients, and especially those in ASA III group, require monitoring and care of an anesthesiologist. Our results showed that propofol provided good sedation and short recovery time. The procedure is rendered painless and no significant respiratory or hemodynamic deteriorations have been observed. Monitoring of blood pressure, heart rate, ECG and oxygen saturation is necessary, as is supplemental administration of oxygen. While some authors recommended that sedation with propofol by nonanesthetists or nurses^[10] are acceptable, we believe that conscious sedation administration and monitoring by an anesthesiologist with an inherent high index of suspicion for potential complications might be a safer strategy.

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Treatment of malignant biliary obstruction by combined percutaneous transhepatic biliary drainage with local tumor treatment

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malignant biliary obstruction, and may improve stent patency.

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Abstract

AIM: To evaluate the utility of local tumor therapy combined with percutaneous transhepatic biliary drainage (PTBD) for malignant obstructive biliary disease.

METHODS: A total of 233 patients with malignant biliary obstruction were treated in our hospital with PTBD by placement of metallic stents and/or plastic tubes. After PTBD, 49 patients accepted brachytherapy or extraradiation therapy or arterial infusion chemotherapy. The patients were followed up with clinical and radiographic evaluation. The survival and stent patency rate were calculated by Kaplan-Meier survival analysis.

RESULTS: Twenty-two patients underwent chemotherapy (11 cases of hepatic carcinoma, 7 cases of pancreatic carcinoma, 4 cases of metastatic lymphadenopathy), and 14 patients received radiotherapy (10 cases of cholangiocarcinoma, 4 cases of pancreatic carcinoma), and 13 patients accepted brachytherapy (7 cases of cholangiocarcinoma, 3 cases of pancreatic carcinoma, 4 cases of metastatic lymphadenopathy). The survival rate of the local tumor treatment group at 1, 3, 6, and 12 months was 97.96%, 95.92%, 89.80%, and 32.59% respectively, longer than that of the non treatment group. The patency rate at 1, 3, 6, and 12 months was 97.96%, 93.86%, 80.93%, and 56.52% respectively. The difference of patency rate was not significant between treatment group and non treatment group.

CONCLUSION: Our results suggest that local tumor therapy could prolong the survival time of patients with

INTRODUCTION

Percutaneous transhepatic biliary drainage (PTBD) is a well-established interventional radiological procedure used in patients with malignant obstructive jaundice, especially in inoperable patients^[1-4]. We treated 233 cases with PTBD from January 1995 to October 2002, 49 of these patients were further treated for local tumors after PTBD. The therapeutic results are reported here.

MATERIALS AND METHODS

From January 1995 to October 2002, 233 consecutive patients (92 females and 141 males, age ranged from 29 to 91 years, mean age of 63.6 years) with malignant biliary obstruction were treated in our hospital with transhepatic placement of metallic stents and/or plastic tubes. The diagnosis and poor prognosis of the patients were established by various imaging methods (US, CT, MRI) and biliary brush cytology or puncture biopsy. Forty-four of these individuals were diagnosed with pancreatic carcinoma, 89 with cholangiocarcinoma, 54 with metastases originating from a variety of primary sites and the remaining 46 with hepatic carcinoma. The obstructing lesion predominantly involved both the right and left main hepatic ducts in 106 patients and the common hepatic and/or proximal common bile duct in 127 patients.

Procedure of percutaneous transhepatic biliary drainage^[3]

After routine percutaneous transhepatic cholangiography,

the obstructing lesions were traversed using Terumo guide-wire techniques. If the guide wire could not pass the stricture, the external catheter was retained. Once the guide wire crossed the lesion and entered the bowel, we could deploy the stent or an internal-external catheter. When a stent was needed, a catheter with a side-arm sheath was employed to inject contrast material over the wire for accurate delineation of the upper and lower margins of the obstructing lesion. After the diagnostic catheter was removed, an 8-10F sheath with a side arm and a homeostasis valve was placed over the wire into the bile duct to allow retrieval of the stent in the event of malfunction of the stent delivery system. The side arm could provide a convenient portal for the injection of contrast material into opacity in the biliary ducts, which could facilitate stent positioning. Eight-millimeter balloons were used in most cases, but 10-mm balloons were required occasionally. Dilation before stent placement as a routine procedure was performed in most of the patients.

Local tumor therapy

Transcatheter arterial chemoembolization (TACE) of all hepatic arteries, celiac and superior mesenteric arteries and their branches should be adequately opacified and all feeding arteries should be carefully identified. We performed TACE initially with infusion of 1000 mg 5-FU, 40~80 mg cisplatin or 200~400 mg carboplatin, 10~20 mg mitomycin or 20~60 mg pharmorubicin, sometimes added 5~15 mL lipiodol with 20~60 mg pirarubicin. We followed up the patients for one or two months with computer tomography, and made TACE 1-3 times.

External irradiation: Cobalt-60 source was used with a total integral dose of 6000cGy at the target length of 1cm, 200cGy per day for 30 d.

Intraluminal brachytherapy: After deployed the stent, we detained a coaxial guider located at the stenosis of endoprosthesis to undergo after-loading treatment. The intraluminal brachytherapy using the high dose rate iridium-192 (¹⁹²Ir) sources was performed to deliver an integral dose of 3000cGy, 500cGy each time, two times a day for 3 d, and standard points were established at 1.0 cm.

Data analysis

Follow-up of each patient was based on outpatient examinations and telephone interviews. The clinical symptoms, phantom change and laboratory examination were recorded. Survival was determined from the time of PTBD. The patent time was defined as the interval between PTBD and obstructive jaundice recurrence. If occlusion did not occur during a patient's life time, the patent period was considered equal to the survival period. Jaundice recurrence was defined as the symptom of jaundice recurred after it subsided and met one of the followings: cholangiography, CT or US demonstrating redilation of bile duct and combined serum bilirubin concentration/total serum bilirubin concentration $\geq 35\%$, or serum bilirubin increased after its obvious drop ($> 100\mu\text{mol/L}$) or after it was lower than half of its original level. The survival and stent patency were calculated by Kaplan-Meier survival analysis. Analysis of data was performed with SPSS/PC version 8.0.

RESULTS

PTBD was successfully performed in all the patients. Stent placement was conducted in 136 cases. Of these 136 patients, 100 were treated with only 1 stent, 20 cases with 2 stents for bilateral drainage and 16 with 2 stents for long strictures. The other 97 patients received plastic catheters. The serum level of total bilirubin reduced from $349.2\pm 155.6\mu\text{mol/L}$ to $178.9\pm 141.2\mu\text{mol/L}$ after PTBD ($t=17.90$, $P=0.000$).

Forty-nine patients (16 females and 33 males, age ranged from 38 to 83 years, a mean age of 64.4 years) accepted local tumor treatment. Twenty-two patients underwent chemotherapy (11 cases of hepatic carcinoma, 7 cases of pancreatic carcinoma, 4 cases of metastasis), one patient died within 1 month because of re-obstruction in bile duct and poor conditions, others received two or three times of treatment for chemical infusion. Fourteen patients received external beam radiation therapy (10 cases of cholangiocarcinoma, 4 cases of pancreatic carcinoma), and 13 patients accepted brachytherapy (7 cases of cholangiocarcinoma, 3 cases of pancreatic carcinoma, 3 cases of metastatic lymphadenopathy). Obstruction occurred at the hilum in 20 cases while 14 cases received multi-drainage, and at the lower common bile duct in 29 cases. Among the 20 patients with obstruction at the hilum, 8 cases received chemotherapy, 6 external beam radiotherapy, and 6 intraluminal brachytherapy. Among the 29 patients with obstruction at the lower common bile duct, 14 cases received chemotherapy, 8 external beam radiotherapy, and 7 intraluminal brachytherapy.

Total serum bilirubin in those who received local tumor treatment reduced from $324.04\pm 166.21\mu\text{mol/L}$ (mean \pm SD) to $87.38\pm 70.96\mu\text{mol/L}$ ($t=11.54$, $P<0.001$). Total serum bilirubin reduced more than 50% in 42 patients and more than 25% in 7 patients. ALT was decreased from $129.3\pm 112.8\text{IU/L}$ to $54.0\pm 48.5\text{IU/L}$ ($t=4.01$, $P<0.001$), GGT was decreased from $576.4\pm 396.9\text{IU/L}$ to $238.8\pm 235.4\text{IU/L}$ ($t=5.58$, $P<0.001$) and ALP was decreased from $687.9\pm 685.1\text{IU/L}$ to $291.5\pm 247.1\text{IU/L}$ ($t=3.47$, $P=0.001$).

Patients with obstruction at the lower common bile duct had more chances to receive local tumor therapy. Stent deployment made it possible for patients to receive further treatment (Table 1).

No severe complication occurred after the procedures. One patient with stent placement died within 1 month because of bile duct re-obstruction and poor conditions. Five patients were still alive during follow-up, and the survival time was 10.5 months (median), and the 1-, 3-, 6- and 12-month survival rate was 97.96%, 95.92%, 89.80%, 32.59%, respectively. Twelve patients who received chemotherapy had re-obstruction during follow-up. Eight patients subsequently developed occluded stents that were treated with one or more additional interventions such as placement of additional stents ($n=2$) or placement of temporal external drainage ($n=6$). Four patients who received catheter drainage had replacement of the plastic catheter. One patient appeared to have distal tumor overgrowth and one exhibited both proximal overgrowth and intraluminal filling defects and three patients developed sludge and debris within

Table 1 Factors of PTBD

| Group | n | Treatment | Chi-square | P-value |
|----------------------|------------|-----------|------------|---------|
| | 184 | 49 | | |
| Hepatic carcinoma | 35 | 11 | | |
| Cholangiocarcinoma | 72 | 17 | | |
| Pancreatic carcinoma | 30 | 14 | | |
| Metastases | 47 | 7 | | |
| Hilar | 107 | 20 | 4.690 | 0.030 |
| CBD | 77 | 29 | | |
| M | 108 | 33 | 1.212 | 0.271 |
| F | 76 | 16 | | |
| Younger than 70 | 132 | 30 | 2.019 | 0.155 |
| Over 70 | 52 | 19 | | |
| Stent | 100 | 36 | 5.822 | 0.016 |
| Catheter | 84 | 13 | | |
| No | 122 | 43 | 8.615 | 0.003 |
| Have | 62 | 6 | | |

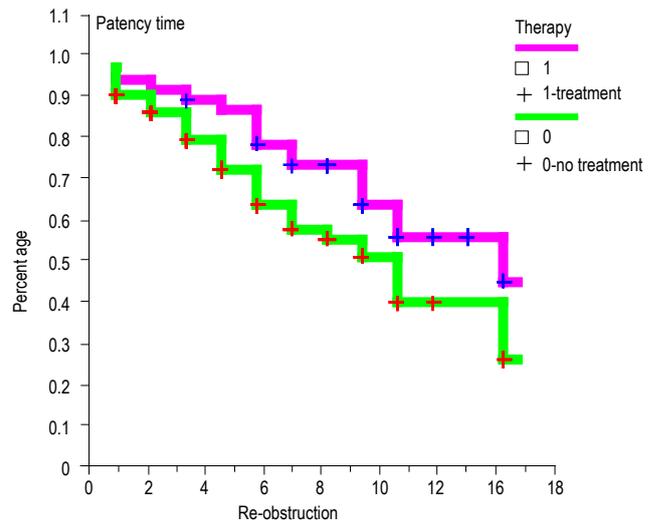


Figure 2 Patency time of drainage (treatment vs non treatment)

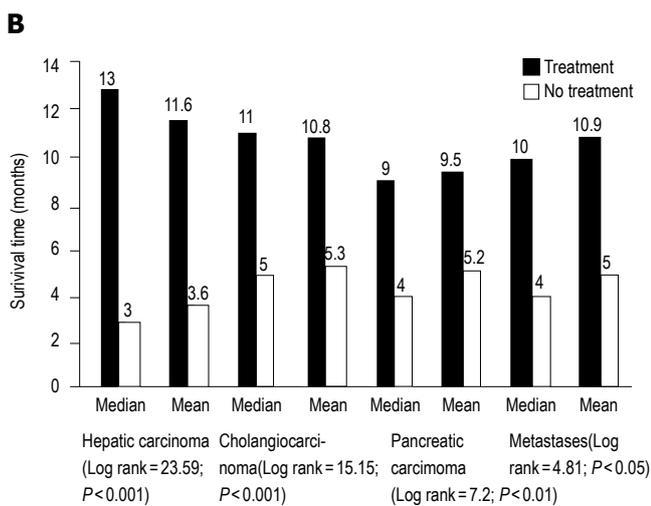
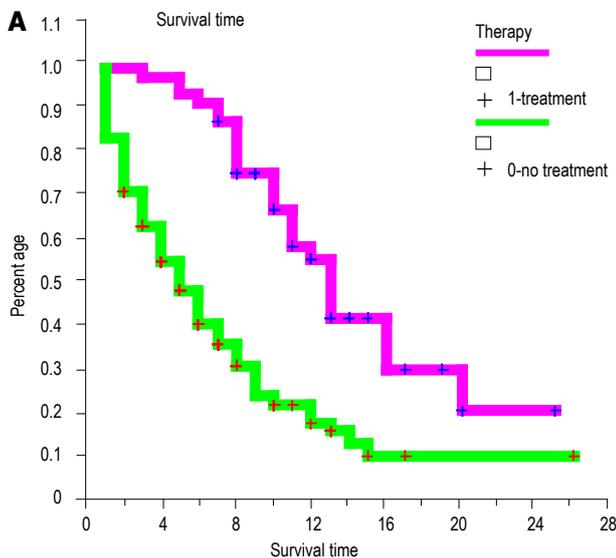


Figure 1 Survival time after treatment (A) in comparison with no treatment (B)

the stent. Seven patients who developed an intraluminal filling defect in stent or catheter did not have any cytological or histological evidence. Two patients who received intraluminal brachytherapy and 2 who received external

irradiation had re-obstruction during follow-up, which was eliminated by balloon dilation. The patency time was 14 mo (median). The patency rate at 1, 3, 6, and 12 mo was 97.96%, 93.86%, 80.93%, and 56.52%, respectively. While the 1-, 3-, 6- and 12- mo survival rate of non-therapy group was 84.24%, 56.97%, 26.40%, and 5.52% respectively, and the patency rate at 1, 3, 6, and 12 months was 94.15%, 84.89%, 66.29%, and 35.73% respectively. The median survival time was significantly longer in the local treatment group than in the no-adjuvant therapy group respectively (Log rank = 46.34, $P < 0.001$) (Figure 1). Though the patency rate of treatment group was higher than that of non-therapy group, there was no significant difference between these two groups (Log rank = 2.38, $P = 0.123$, Figure 2)

DISCUSSION

Surgical therapy of malignant obstructive jaundice includes a curative resection or a palliative operation of the tumor. Unfortunately, the surgical cure rate of pancreatic, bile duct and gall bladder carcinoma, and hepatic carcinoma is lower than 5%^[5,6]. Since the resectability of pancreatic cancer remains below 30%^[7], palliative treatment is recommended for patients with unresectable tumor. Interventional radiologists play an important role in the management of patients with malignant biliary obstruction. Biliary drainage is usually used as the initial treatment because of overt cholangitis. All procedures were performed using fluoroscopic guidance alone in our study. Early and effective biliary drainage might be necessary in this group of patients with limited hepatic function to improve the prognosis^[8]. PTBD for inoperable malignant obstructive jaundice is a widely used palliative procedure^[1-3].

Yan^[9] pointed out that bile drainage as a palliative therapy for malignant obstructive jaundice could not prolong the survival time, but can improve life quality. Successful biliary drainage after biliary decompression is associated with improvement in quality of life^[10]. However, PTBD could provide access to the intrahepatic and extrahepatic bile ducts^[11-13].

Since PTBD alleviates icterus and improves liver reserve function, patients with tumor could have more chances to receive chemotherapy or radiotherapy or photodynamic therapy^[14] or radiofrequency ablation^[15]. But how to choose system chemotherapy and artery infusion chemotherapy may be a question. Xia and Wang^[16] reported that intra-artery treatment is more effective than system chemotherapy. Also radiotherapy can improve the survival^[17]. Huang *et al*^[18] demonstrated that radiotherapy is an effective adjuvant strategy in those who have a limited response to TACE or have a poor liver reserve function.

Occlusion of the stent by overgrowth of proximal or distal ends can be easily demonstrated and identified. In some cases, the defects consist of desquamated cells, mucus, and sludge. It has been speculated that these defects may be caused by tumor ingrowth through the meshes of the stent^[19]. Drug infusion via supplying artery and embolization therapy have been used in clinical practice as an effective way of suppressing the growth of malignant tumors^[20-23]. Further tumor therapy may improve the patency of stent and survival^[24-26].

TACE therapy could increase common bile duct obstruction due to tumor thrombi^[27]. External beam radiation therapy may be beneficial in some patients with unresectable icteric-type HCC^[28, 29]. When combined with other conventional therapies (such as TACE), radiation therapy may play an important role in the treatment of HCC^[30, 31].

In conclusion, PTBD is an effective palliative procedure for improving malignant obstructive jaundice.

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CASE REPORT

Reversion of severe hepatopulmonary syndrome in a non cirrhotic patient after corticosteroid treatment for granulomatous hepatitis: A case report and review of the literature

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Abstract

Hepatopulmonary syndrome (HPS) is defined as a clinical triad including liver disease, abnormal pulmonary gas exchange and evidence of intrapulmonary vascular dilatations. We report a 61-year-old male presented with fatigue, long-lasting fever, loss of weight, signs of portal hypertension, hepatosplenomegaly, cholestasis and progressive dyspnoea over the last year. Clinical, laboratory and histological findings confirmed the diagnosis of granulomatous hepatitis. HPS due to hepatic granuloma-induced portal hypertension was proved to be the cause of severe hypoxemia of the patient as confirmed by contrast-enhanced echocardiography. Reversion of HPS after corticosteroid therapy was confirmed by a new contrast-enhanced echocardiography along with the normalization of cholestatic enzymes and improvement of the patient's conditions. This is the first case of complete reversion of HPS in a non-cirrhotic patient with hepatic granuloma, indicating that intrapulmonary shunt in liver diseases is a functional phenomenon and HPS can be developed even in miscellaneous liver involvement as in this case.

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Key words: Hepatopulmonary syndrome; Granulomatous hepatitis; Liver cirrhosis; Orthodeoxia; Platypnoea

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INTRODUCTION

Hepatopulmonary syndrome (HPS) is defined as the triad of advanced liver disease, hypoxemia ($P_{aO_2} < 70$ mmHg or increased alveolar-arterial oxygen gradient > 20 mmHg) while breathing room air, and evidence of intrapulmonary vascular dilatations (IPVDs) resulting in an excess perfusion for a given state of ventilation irrespective of the presence or absence of cardiopulmonary disease^[1,2]. HPS is a quite common condition in patients with advanced liver diseases as it has been reported in approximately 10-20% of candidates for liver transplantation^[3]. The incidence rate of IPVDs in patients with end-stage liver disease is 13-47%^[4] and 10% in normoxemic patients with early liver cirrhosis^[5]. However, the prevalence of HPS according to the above mentioned criteria ranges from 5% to 30%^[2,6,7]. Though the majority of patients with HPS have developed cirrhosis and portal hypertension, the presence of HPS is occasionally reported in cases of portal hypertension without the existence of cirrhosis. Actually, HPS has been reported in patients with Budd-Chiari syndrome^[8] and acute^[9] or chronic viral hepatitis^[4].

In this paper, we describe a non-cirrhotic patient with granulomatous hepatitis presented with signs of HPS, which responded completely to corticosteroid administration.

CASE REPORT

A 61-year-old man was admitted to our department because of low-grade fever (up to 38°C), weakness, fatigability, loss of weight (10 kg during the last 8 mo) and progressive dyspnoea over the last year. The patient reported pruritus in the last 20 d and low exercise tolerance. He was an

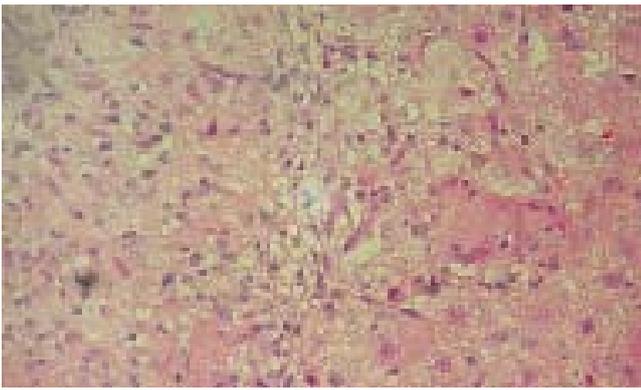


Figure 1 Liver histopathology shows an epithelioid granuloma without central necrosis. The granuloma is consisted of epithelioid macrophages and some chronic inflammatory cells. There is also a multinucleated giant cell (Hematoxylin and eosin staining x 40).

ex-smoker (80 packs a year) until 8 months earlier and had a 40-year history of alcohol abuse (over 100g of alcohol daily).

On admission, he was pale and well orientated, his temperature was 37.4°C, his pulse rate was 60 per minute and his blood pressure was 110/60 mmHg. Physical examination revealed profound dyspnoea with a high respiratory rate (30 per minute), platypnea and cyanosis, jaundice, hepatosplenomegaly without the presence of ascites, palmar erythema, digital clubbing and extended cutaneous spider naevi on the upper half of the body. Pulmonary auscultation and heart sounds were normal without murmurs or gallops. Further physical examination was unremarkable.

Laboratory test on admission showed mild normochromic-normocytic anaemia with 35% haematocrit, 118g/L haemoglobin, 3.48×10^{10} /L reticulocytes and normal platelet count (16.8×10^9 /L). White blood cells were 3.5×10^9 /L with 57.4% segmented granulocytes, 29.5% lymphocytes and 9.9% monocytes. Peripheral blood smear was normal. Abnormal biochemical parameters were 67 mg/dL urea, 1.47 mg/dL creatinine, 11.0 mg/dL serum calcium, 3.2 mg/dL total bilirubin, 1.26 mg/dL direct bilirubin, 348 U/L alkaline phosphatase (ALP) (upper normal <104 U/L), 516 U/L γ -glutamyltranspeptidase (γ -GT) (upper normal <40 U/L), 73U/L aspartate aminotransferase (AST) (upper normal <40U/L) and 43U/L alaninoaminotransferase (ALT) (upper normal <40 U/L). Urinalysis revealed specific gravity of 1.010, pH 6.0, 1-2 red blood cells/HPF, 0-1 white blood cell/HPF, without casts, 760 mg/24h proteinuria and elevated urine calcium excretion (326 mg/24h) accompanied with reduced creatinine clearance (38.7mg/dL). Electrocardiography and chest X-ray radiography were normal. Upper gastrointestinal endoscopy revealed first-degree oesophageal varices and mild portal gastropathy.

Sequential bacterial cultures of blood, urine and marrow were negative for tuberculosis bacilli, brucellosis or other bacterial and fungal infectious diseases. Serology for hepatitis A, B, C and human immunodeficiency virus infections was negative. Abdominal ultrasound did not reveal lymphadenopathy, ascites or hepatic nodules.

Abdominal dynamic spiral computed tomography (CT) confirmed the presence of hepatosplenomegaly. Serum immunoglobulin M was mildly elevated (269mg/dL; upper normal limit: 249mg/dL) but antimicrobial antibodies by ELISA, Western blot and indirect immunofluorescence (IIF) using commercial and in-house substrates were negative. Antinuclear autoantibodies using IIF were also negative. Liver biopsy revealed multiple non-caseating epithelioid granulomas and mild perisinusoidal fibrosis without any histological sign of cirrhosis (Figure 1). Multiple non-caseating granulomas were also found in the bone marrow biopsy specimen. High resolution CT of the chest was normal. Bronchoalveolar lavage fluid revealed the presence of lymphocytes mainly T-cells in 94% with a CD4/CD8 ratio of 2. Transbronchial biopsy was negative for granulomas. The above clinical, laboratory, histological and radiological findings indicated that idiopathic granulomatous hepatitis was the most likely diagnosis though the diagnosis of systemic sarcoidosis without apparent involvement of the lungs could not be rule out.

Arterial blood gas analysis (while breathing room air) showed hypoxemia with arterial oxygen tension (P_{aO_2}): 53 mmHg; P_{aCO_2} : 27.9 mmHg; pH: 7.48; bicarbonate: 21mM; PA-aO₂: 66.7 mmHg. Supplemental 100% oxygen was administered and P_{aO_2} was elevated to 153 mmHg. Arterial blood gases while breathing room air became worse at the upright position (P_{aO_2} : 49 mmHg, P_{aCO_2} : 23.9 mmHg, pH: 7.42, bicarbonate: 15.5mmol/L) while improved at supine position. The observed orthodeoxia was compatible to the clinical improvement noted during supine position (platypnea). Spirometry revealed findings of chronic obstructive pulmonary disease of stage I, according to Global Initiative for Chronic Obstructive Lung Diseases (GOLD) classification^[10]: FVC: 4.14 lt (110 % pred), FEV1: 2.68 lt (90 % pred), FEV1/FVC: 65%, FEF25-75%: 1.43 L/sec (42 % pred). The diffusion capacity test showed a moderately reduced DL, CO/VA: 0.75 (56% pred).

Contrast-enhanced echocardiography with agitated saline showed microbubble opacification in the left atrial after six heart beats later of the right heart chamber opacification in the absence of intracardiac septal defects, confirming the presence of intrapulmonary shunt (Figures 2A and 2B). Technetium 99m-labelad macroaggregated albumin scanning was also positive for IPVDs (increased uptake of radionuclide over the kidneys and brain), confirming the diagnosis of HPS on the basis of portal hypertension due to hepatic granulomas.

The patient started to receive methylprednisolone (32 mg daily) and continuous home oxygen therapy (2L/min) for at least 18 hours per day. Three months later, his performance status was excellent and arterial blood gas analysis (while breathing room air) was almost normal (P_{aO_2} : 85 mmHg, P_{aCO_2} : 36.2 mmHg, pH: 7.43, bicarbonate: 25.4mmol/L with PA-aO₂ 19.7 mmHg). Thirteen months after his first evaluation, he was still on methylprednisolone (4 mg daily). His performance status was very good with his blood gas, liver function and calcium concentration within normal limits. In addition, after treatment the spleen was not palpable, a new upper gastrointestinal endoscopy was normal and a new contrast-enhanced echocardiography revealed no intrapulmonary shunt (Fig-

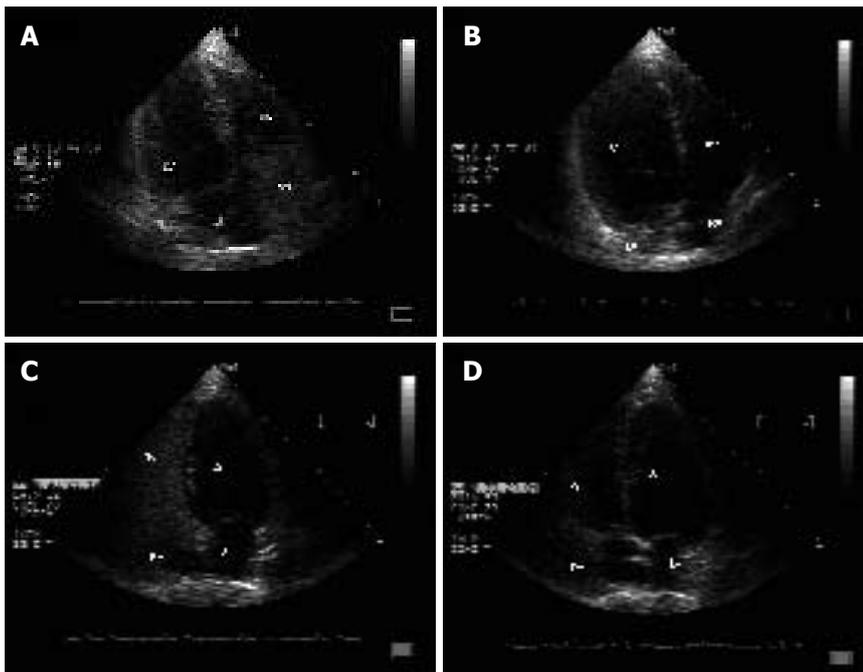


Figure 2 Contrast-enhanced echocardiography image of the patient before (A, B) and after (C, D) corticosteroid treatment for granulomatous hepatitis. A, C: Microbubble appearance in the right heart chamber (RA and RV) after the bolus infusion of agitated saline; B: Microbubble opacification in the left heart chambers (LA and LV) is not detected after six to eight heart beats; D: Microbubble opacification in the left heart chambers (LA and LV) is not detected after six to eight heart beats.

ures 2C and 2D).

DISCUSSION

This case report describes a patient with HPS due to portal hypertension as a result of granulomatous hepatitis. Though the most common causes of granulomatous hepatitis are infections and systemic sarcoidosis, we were not able to confirm such a diagnosis (due to the absence of lung involvement). Indeed, liver involvement in sarcoidosis is not unusual as 50-80% of patients are affected but almost all of them have also lung disease^[11]. However, to the best of our knowledge, this is the first case of complete reversion of HPS after corticosteroid treatment in a non-cirrhotic patient suffering from portal hypertension due to idiopathic granulomatous hepatitis.

HPS is a well-known complication in patients with advanced liver disease. Its major signs and symptoms include the presence of dyspnoea (particularly in exertion), cyanosis, digital clubbing, platypnea and orthodeoxia (a decrease in P_{aO_2} of more than 3 mmHg when the patient moves from the supine to the standing position)^[12-14]. The pathogenesis of HPS is well delineated^[1]. The syndrome occurs as a result of precapillary and capillary pulmonary vascular dilatations, causing apparent right-to-left intrapulmonary shunting, though there is no true anatomic shunt^[15, 16]. However, the capillaries may become dilated to 500 μm in diameter (normal range 8-15 μm)^[16, 17]. Hypoxemia arises due to a combination of ventilation and perfusion mismatching, oxygen diffusing limitation and intrapulmonary shunting^[13, 16, 18]. These intrapulmonary vascular abnormalities in advanced liver cirrhosis are attributed to an excess production of vasodilators that affect the lung vascular system via porto-systemic shunt, with nitric oxide as the most likely mediator^[19]. The above hypothesis is in accordance with the complete reversion of the syndrome when liver transplantation is successful in patients with end-stage

liver disease and HPS^[12, 15, 16, 19].

However, it was reported that HPS occurs occasionally in non-cirrhotic cases of portal hypertension^[4, 8, 9] and could completely reverse after the causative agent is eradicated^[8, 9]. Under this context, we believe that in our case the corticosteroid treatment of granulomatous hepatitis led to the reversion of HPS via an improvement of portal hypertension as the presence of liver granuloma per se can result in the development of sinusoidal portal hypertension irrespective of the presence or absence of cirrhosis.

Staging of the severity of HPS is important because severity influences survival and is useful in determining the timing and risks for liver transplantation. A classification of the severity of HPS based on oxygenation abnormalities in four stages has been proposed^[1, 6]. According to this classification our patient had severe HPS as he presented with 49 mmHg P_{aO_2} while breathing room air, 153 mmHg P_{aO_2} when supplemental oxygen was admitted and a significant elevation of $PA-aO_2$. Contrast-enhanced echocardiography is the standard method for the diagnosis of HPS^[12]. Technetium 99m-labeled macroaggregated albumin scanning is another method of detecting IPVDs^[20] and can be used to quantify the magnitude of shunting.

In conclusion, to the best of our knowledge this is the first case study to describe complete reversion of HPS due to granulomatous hepatitis. We consider that the clinical and laboratory improvement of severe HPS in this case is due to the treatment response, leading to an improvement of portal hypertension as attested by the complete normalization of cholestatic enzymes, the absence of oesophageal varices and the shrinkage of spleen after corticosteroid administration. The absence of cirrhosis proved by liver biopsy may be a determinant factor for the reversion of HPS. It seems that HPS can be reversed (alleviation of dyspnea, improvement of hypoxemia) when liver function and portal hypertension are improved.

This further supports the fact that the development of intrapulmonary shunt in liver diseases is mainly a functional phenomenon and HPS can be observed even in miscellaneous liver involvement as in this case.

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Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as γ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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Use SI units. For example: body mass, *m* (B) = 78 kg; blood pressure, *p*(B) = 16.2/12.3 kPa; incubation time, *t* (incubation) = 96 h, blood glucose concentration, *c* (glucose) 6.4 \pm 2.1 mmol/L; blood CEA mass concentration, *p* (CEA) = 8.6 24.5 μ g/L; CO₂ volume fraction, 50 mL/L CO₂ not 5% CO₂; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, etc. Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

Genotypes: *gyrA*, *arg 1*, *c myc*, *c fos*, etc.

Restriction enzymes: *EcoRI*, *HindI*, *BamHI*, *Kho I*, *Kpn I*, etc.

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World Journal of Gastroenterology standard of quantities and units

| Number | Nonstandard | Standard | Notice |
|--------|--|--|--|
| 1 | 4 days | 4 d | In figures, tables and numerical narration |
| 2 | 4 days | four days | In text narration |
| 3 | day | d | After Arabic numerals |
| 4 | Four d | Four days | At the beginning of a sentence |
| 5 | 2 hours | 2 h | After Arabic numerals |
| 6 | 2 hs | 2 h | After Arabic numerals |
| 7 | hr, hrs, | h | After Arabic numerals |
| 8 | 10 seconds | 10 s | After Arabic numerals |
| 9 | 10 year | 10 years | In text narration |
| 10 | Ten yr | Ten years | At the beginning of a sentence |
| 11 | 0,1,2 years | 0,1,2 yr | In figures and tables |
| 12 | 0,1,2 year | 0,1,2 yr | In figures and tables |
| 13 | 4 weeks | 4 wk | |
| 14 | Four wk | Four weeks | At the beginning of a sentence |
| 15 | 2 months | 2 mo | In figures and tables |
| 16 | Two mo | Two months | At the beginning of a sentence |
| 17 | 10 minutes | 10 min | |
| 18 | Ten min | Ten minutes | At the beginning of a sentence |
| 19 | 50% (V/V) | 500 mL/L | |
| 20 | 50% (m/V) | 500 g/L | |
| 21 | 1 M | 1 mol/L | |
| 22 | 10 μM | 10 μmol/L | |
| 23 | 1N HCl | 1 mol/L HCl | |
| 24 | 1N H ₂ SO ₄ | 0.5 mol/L H ₂ SO ₄ | |
| 25 | 4rd edition | 4 th edition | |
| 26 | 15 year experience | 15- year experience | |
| 27 | 18.5 kDa | 18.5 ku, 18 500u or M:18 500 | |
| 28 | 25 g.kg ⁻¹ /d ⁻¹ | 25 g/(kg·d) or 25 g/kg per day | |
| 29 | 6900 | 6 900 | |
| 30 | 1000 rpm | 1 000 r/min | |
| 31 | sec | s | After Arabic numerals |
| 32 | 1 pg L ⁻¹ | 1 pg/L | |
| 33 | 10 kilograms | 10 kg | |
| 34 | 13 000 rpm | 13 000 g | High speed; g should be in italic and suitable conversion. |
| 35 | 1000 g | 1 000 r/min | Low speed. g cannot be used. |
| 36 | Gene bank | GenBank | International classified genetic materials collection bank |
| 37 | Ten L | Ten liters | At the beginning of a sentence |
| 38 | Ten mL | Ten milliliters | At the beginning of a sentence |
| 39 | umol | μmol | |
| 40 | 30 sec | 30 s | |
| 41 | 1 g/dl | 10 g/L | 10-fold conversion |
| 42 | OD ₂₆₀ | A ₂₆₀ | "OD" has been abandoned. |
| 43 | One g/L | One microgram per liter | At the beginning of a sentence |
| 44 | A260 nm ^b P<0.05 | A ₂₆₀ nm ^a P<0.05 | A should be in italic. In Table, no note is needed if there is no significance instatistics: ^a P<0.05, ^b P<0.01 (no note if P>0.05). If ther is a second set of P value in the same table, ^c P<0.05 and ^d P<0.01 are used for a third set: ^a P<0.05, ⁱ P<0.01. |
| 45 | [*] F=9.87, [§] F=25.9, [#] F=67.4 | ¹ F=9.87, ² F=25.9, ³ F=67.4 | Notices in or under a table |
| 46 | KM | km | kilometer |
| 47 | CM | cm | centimeter |
| 48 | MM | mm | millimeter |
| 49 | Kg, KG | kg | kilogram |
| 50 | Gm, gr | g | gram |
| 51 | nt | N | newton |
| 52 | l | L | liter |
| 53 | db | dB | decibel |
| 54 | rpm | r/min | rotation per minute |
| 55 | bq | Bq | becquerel, a unit symbol |
| 56 | amp | A | ampere |
| 57 | coul | C | coulomb |
| 58 | HZ | Hz | |
| 59 | w | W | watt |
| 60 | KPa | kPa | kilo-pascal |
| 61 | p | Pa | pascal |
| 62 | ev | EV | volt (electronic unit) |
| 63 | Jonle | J | joule |
| 64 | J/mmol | kJ/mol | kilojoule per mole |
| 65 | 10×10×10cm ³ | 10 cm×10 cm×10 cm | |
| 66 | N·km | KN·m | moment |
| 67 | x±s | mean±SD | In figures, tables or text narration |
| 68 | Mean±SEM | mean±SE | In figures, tables or text narration |
| 69 | im | im | intramuscular injection |
| 70 | iv | iv | intravenous injection |
| 71 | Wang et al | Wang <i>et al.</i> | |
| 72 | EcoRI | EcoRI | <i>Eco</i> in italic and RI in positive. Restriction endonuclease has its prescript form of writing. |
| 73 | Ecoli | <i>E.coli</i> | Bacteria and other biologic terms have their specific expression. |
| 74 | Hp | <i>H pylori</i> | |
| 75 | Iga | Iga | writing form of genes |
| 76 | igA | IgA | writing form of proteins |
| 77 | ~70 kDa | ~70 ku | |

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Non-alcoholic fatty liver disease and the metabolic syndrome: Effects of weight loss and a review of popular diets. Are low carbohydrate diets the answer?

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Abstract

Non-alcoholic fatty liver disease (NAFLD) encompasses a wide spectrum of fat-induced liver injury, ranging from relatively benign steatosis to cirrhosis and liver failure. The presence of obesity and insulin resistance is strongly associated with non-alcoholic fatty liver and confers on it a greater risk of histologically advanced disease. There is a growing concern in the medical profession as the prevalence of this disease continues to rise in parallel with the rise in obesity and the metabolic syndrome. Treatment options are limited and dietary weight loss is often advised. Low fat diets are difficult to adhere to and recent studies have shown the potential of low carbohydrate diets for weight loss and improving insulin resistance. Thus far, no study has evaluated the effect of low carbohydrate diets on NAFLD. Future studies will be required to address this question and others with regards to the nutritional adequacy and long-term side effects of these diets.

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Key words: Non-alcoholic fatty liver disease; Obesity; Metabolic syndrome; Diet management

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INTRODUCTION

In the 1970s, patients undergoing jejunioileal bypass surgery

for morbid obesity were noted to develop steatohepatitis and even liver failure following rapid weight loss^[1]. Their liver histology was similar to that seen in alcoholics, with macrovesicular steatosis, Mallory hyaline, focal hepatocyte necrosis, mixed lobular inflammation and fibrosis^[2]. Similar findings were later described in obese patients without significant alcohol consumption. In 1980, Ludwig *et al*^[3] coined the term 'non-alcoholic steatohepatitis' (NASH) to describe these findings. Since then, interest in the disease has grown exponentially in keeping with its rising prevalence. NASH was initially thought to be a benign condition largely limited to middle-aged obese women with diabetes. However, recent studies have shown it to be a far more complex disease that is found in men, women and even children. The spectrum of disease ranges from pure steatosis alone to NASH with hepatic fibrosis, cirrhosis, hepatocellular carcinoma, liver failure and even death^[4-6]. Estimates suggest that 20-30% of adults in Western countries may have NAFLD and about 10% of these individuals meet criteria for diagnosis of NASH^[7]. NAFLD is now recognized as the most common cause of chronically elevated liver transaminases^[8,9] and may be the most common liver disorder^[10]. Obesity is the single most common condition found in association with NAFLD. Other features of the metabolic syndrome, such as hyperinsulinemia, hypertriglyceridemia and hypertension, also play a significant pathophysiologic role in its development.

In general, NAFLD in the absence of NASH is an indolent disease with a benign course. However, as noted, end stage liver disease may occur as a consequence of NASH. The seriousness of this condition is demonstrated by the fact that approximately 50% of patients develop fibrosis, 15% develop cirrhosis and 3% may advance to liver failure requiring transplantation^[11]. NASH is now being recognized as the underlying cause of most cases of cryptogenic cirrhosis^[12,13]. The natural history of NAFLD is poorly understood, and it is not known why some patients progress to cirrhosis, while others do not. However, obesity and insulin resistance have been shown to be associated with more histological advanced disease.

The aim of this article is to review the role of the metabolic syndrome, especially insulin resistance and obesity in the development of NAFLD, to discuss the effect of weight loss on NAFLD and, finally, to evaluate popular diets and compare them with regard to their effects on the metabolic syndrome and NAFLD.

NASH AND METABOLIC SYNDROME

Fatty liver is commonly associated with obesity and insulin resistance. The increasing incidence of NAFLD closely parallels these conditions. There is abundant data showing a relationship between obesity and NAFLD. Wanless *et al*^[14] in an autopsy study of 351 patients found that 70% of obese patients had liver steatosis, and the degree of steatosis was proportional to the degree of obesity. The authors also found steatohepatitis in 18.5% and severe fibrosis in 13.8% of markedly obese patients, compared to steatohepatitis in 2.7% and severe fibrosis in 6.6% of lean people. A prospective study performed by Klain *et al*^[15] evaluated liver biopsies from 100 consecutive morbidly obese patients undergoing Roux-en-Y gastric bypass. Histological abnormalities were found in 98% of biopsies, and ranged from mild fatty infiltration through inflammatory change to fibrosis and cirrhosis^[15]. Data from 90 patients with NASH demonstrated insulin resistance in 85% of them^[16]. An Italian study evaluated the risk factors associated with hepatic steatosis. A total of 257 participants were assigned to one of four categories: Controls, teetotalers with normal body mass index (BMI); obese teetotalers; heavy drinkers (> 60 g of alcohol per day) with normal BMI; and heavy drinkers with obesity. The prevalence of steatosis on ultrasound increased from 16% in controls to 46% in heavy drinkers, 76% in obese individuals and 95% in patients with both obesity and heavy alcohol intake. Compared with controls, steatosis was more common by 2.8-fold in heavy drinkers, 4.6-fold in obese persons and 5.8 fold in obese heavy drinkers. In heavy drinkers, obesity increased the risk of steatosis 2.0-fold, while heavy drinking was associated with only a 1.0-fold increased risk in obese subjects^[17]. The authors concluded that steatosis was more strongly associated with obesity than with heavy drinking.

Evidence of an etiologic association between NAFLD and metabolic syndrome (hyperglycemia, central obesity, hypertension, hypertriglyceridemia and low HDL-cholesterol) has been shown in both obese and non-obese patients^[18]. Studies also have shown that patients with NASH are more insulin resistant than patients with fatty liver alone^[19]. Given the wealth of data supporting it, many researchers now consider NAFLD to be a hepatic manifestation of the metabolic syndrome, instead of a primary liver disease^[16,20]. Chitturi *et al*^[21] tested the hypothesis that insulin resistance is an essential requirement for the development of NASH, and that a high association between insulin resistance and liver disease is relatively specific for NASH. Sixty-six patients with NASH were studied. Insulin resistance was found in virtually all patients (98%) and was seen in both lean and overweight patients. A subset of 36 patients with less severe NASH were compared to 36 age- and sex-matched patients with chronic hepatitis C. The prevalence of insulin resistance was significantly higher in those with NASH than in comparable cases of HCV (75% *vs* 8.3%)^[21]. Marchesini *et al*^[19] studied liver biopsies in patients with NAFLD. Based on histology, these were classified as having NASH *vs* pure fatty liver. The investigators found that 88% of patients with NASH had metabolic syndrome

compared with 53% in patients with pure fatty liver^[19]. Marceau *et al*^[22] investigated the relationship between liver pathology and the metabolic syndrome. Five hundred fifty one severely obese patients undergoing anti-obesity surgery were studied. Steatosis was found in 86%, fibrosis in 74%, steatohepatitis in 24% and unexpected cirrhosis in 2%. With each addition of the components of metabolic syndrome, the risk of steatosis increased exponentially from 1- to 99-fold^[22]. In a series of 505 severely obese patients evaluated before gastroplasty, prevalence of steatosis was significantly higher in patients with impaired glucose tolerance or type II diabetes as compared with non-diabetics. The severity of steatosis was positively correlated with BMI, fasting plasma glucose, insulin and triglyceride concentrations, as well as serum ALT, AST and GGT levels^[23].

Issues regarding the nature of hyperinsulinemia in NASH have been raised. It has been questioned as to whether hyperinsulinemia and insulin resistance occur as part of the metabolic syndrome or whether liver damage itself leads to chronic hyperinsulinemia and insulin resistance from impaired insulin degradation, as is seen in cirrhosis. Chitturi *et al*^[21] compared the patients with NASH and mild or absent fibrosis with age- and sex-matched patients with HCV, and found that the patients with NASH showed more attributes of insulin resistance than the controls. They had much higher levels of insulin resistance, serum insulin and C-peptide levels. However, the serum C-peptide/insulin ratio was similar in both groups^[21]. Pagano *et al*^[24] addressed the same question, comparing 19 patients with histologically mild NASH, who had functionally competent livers with 19 normal subjects. Patients with NASH showed marked hyperinsulinemia and insulin resistance as compared with controls, however, the hepatic insulin extraction was similar in both groups^[24]. These two studies showed that insulin hypersecretion, and not just impaired insulin degradation, was the basis for hyperinsulinemia in NASH.

The overall incidence of NASH in the severely obese is reported to range from 25-36.4%^[25-27]. The prevalence of obesity in the Western world has shown a large increase in the last 20 years. The data of the National Health and Nutrition Examination Survey (NHANES II, 1976-1980) showed a prevalence of 14.5%. By NHANES III (1988-1994), this number had increased to 22.5%, and the data of NHANES 1999-2000 showed a prevalence of 30.5%^[28]. Significantly, this number could reach 40% by the year 2025^[29]. A similar increase in the number of patients with type 2 diabetes is expected. By some estimates, 29 million people or 7.2% of the population will have type 2 diabetes by the year 2050^[30]. Of grave concern is the increasing incidence of obesity in children and adolescents. Given these statistics, the incidence of NASH will rise significantly in the coming years and so will hepatic complications arising from it.

Factors responsible for the development of NAFLD in obese patients are not clear, and the exact mechanism of its progression to fibrosis and cirrhosis has yet to be elucidated. However, our understanding of disease pathogenesis has advanced significantly. Insulin resistance is thought to be a primary pathophysiologic mechanism

in development of fatty liver. Current understanding of the pathogenesis is as follows: Insulin resistance and visceral obesity lead to a hepatic influx of free fatty acids, resulting in increased triglyceride synthesis and decreased triglyceride export. This leads to hepatic steatosis. At this stage, patients have the relatively benign condition of NAFLD. Some of these patients will go on to steatohepatitis. It is unclear why only a small fraction will advance to NASH and what is the exact impetus for this advance. One proposal is that these lipid-laden hepatocytes are susceptible to a “second-hit”^[31]. The exact mechanism of this second-hit is unknown. In NASH, as in alcoholic hepatitis, oxidative stress and lipid peroxidation have emerged as the most likely candidates. This “hit” occurs via increased mitochondrial beta-oxidation of the free fatty acids, production of reactive oxygen species and depletion of antioxidants glutathione and vitamin E. This depletion of anti-oxidants hampers reactive oxygen species inactivation, and increases the deleterious effects on the mitochondria. Oxidative stress also results in abnormal cytokine production, especially TNF- α through up-regulation of nuclear translocation of transcription factor nuclear factor κ B. This combination of lipid peroxidation and cytokine production results in hepatocyte death.

Another proposed mechanism of development of NASH includes a primary mitochondrial abnormality, as proposed by Sanyal *et al*^[32]. This defect, otherwise clinically silent, leads to increased mitochondrial beta oxidation and production of reactive oxygen species in the presence of insulin resistance.

Yang *et al*^[33] have demonstrated that obesity itself may cause progression to steatohepatitis by causing Kupffer cell dysfunction and sensitizing the hepatocytes to endotoxin, suggesting that the progression of liver disease may depend on the extent of fatty infiltration^[33].

Iron, a strong oxidative agent, has also been proposed as a factor causing the second-hit. Elevated serum ferritin and insulin resistance on those levels have been noted in patients with NASH, as well as increased prevalence of C282Y and H63D mutations in the HFE gene^[34,35]. However, evidence that hepatic insulin resistance plays a significant role in fibrosis was found in only one study, and recent studies suggested that increased ferritin levels were likely markers of severe histologic damage and not iron overload^[36]. Leptin production by activated hepatic stellate cells has also been considered an important factor in the progression of fatty liver disease and development of fibrosis^[37]. Supporting evidence is furnished by genetically leptin-deficient ob/ob mice, which do not develop fibrosis even when fed a methionine-choline-deficient diet.

OBESITY AND INSULIN RESISTANCE AS PREDICTORS OF FIBROSIS

The natural history of NAFLD is not well known, but it is known that prognosis varies according to histologic type. Matteoni *et al*^[5] conducted a retrospective study to compare clinical characteristics and outcomes of patients with different types of NAFLD. Patients were separated into four histologic types: Simple fatty liver; steatohepatitis;

steatonecrosis; and steatonecrosis plus either Mallory hyaline or fibrosis. Cirrhosis and liver-related death were seen almost exclusively in patients with steatonecrosis with or without Mallory hyaline or fibrosis^[5]. The study also confirmed that the prognosis of simple steatosis is favorable.

A number of risk factors for more histologically advanced disease have been identified. These include central weight distribution and metabolic syndrome. Dixon *et al*^[38] studied 105 severely obese individuals undergoing bariatric surgery, and showed that hyperinsulinemia and increased insulin resistance were associated with adverse histologic findings. The study found that C-peptide was the best predictor of advanced fibrosis (stage 3-4) and that patients with advanced fibrosis had significantly higher C-peptide levels. The insulin resistance index and systemic hypertension were independently associated with advanced NAFLD. Insulin resistance was found to be the best predictor of zone 3-centric steatosis, inflammation and fibrosis. Interestingly, this study found that moderate alcohol consumption reduced the risk of NAFLD by decreasing insulin resistance. Angulo *et al*^[39] studied liver biopsies from 144 patients with NASH. In multivariate analysis, older age (>45 years), obesity, diabetes mellitus and an AST/ALT ratio >1 were significant predictors of severe fibrosis (bridging/cirrhosis). The investigators concluded that this subgroup would benefit most from liver biopsy and investigational therapies^[39]. Researchers in France investigated 93 obese patients, and found that age \geq 50 years, BMI >28 kg/m², triglycerides >1.7 mmol/L and ALT >2 times normal value were independently associated with septal fibrosis^[40]. A univariate analysis showed that diabetes and impaired glucose tolerance were significantly associated with fibrosis. Another recent study evaluating steatosis in chronic hepatitis C found that an increased BMI had a role in pathogenesis of steatosis in chronic hepatitis C, and that this may contribute to fibrosis^[41]. Studies by both Marceau *et al*^[22] and Willner *et al*^[16] showed that patients with cirrhosis were more obese than those without cirrhosis. In addition, Marceau *et al*^[22] found the presence of diabetes to be the strongest predictor of cirrhosis. Finally, a recent investigation of NAFLD and the metabolic syndrome showed that the presence of metabolic syndrome carried a high risk for NASH among NAFLD patients, and was also associated with a high risk of severe fibrosis^[19].

Thus, features of the metabolic syndrome like obesity, insulin resistance and hypertriglyceridemia are not only predisposing factors for NASH, but are also risk factors for more severe fibrosis and advanced disease.

TREATMENT OPTIONS

In light of the increasing incidence of NAFLD-associated comorbid conditions and NAFLD itself, as well as increased awareness of adverse outcomes associated with steatohepatitis, a number of treatment options are being explored. These combine specific therapies for NAFLD as well as the management of comorbid conditions. Therapies that have been evaluated include lifestyle changes such as

diet and exercise, antioxidants like vitamin E and betaine, cytoprotective agents such as ursodeoxycholic acid, lipid-lowering agents, anti-diabetics, weight-loss agents like orlistat and iron reduction therapy, i.e. phlebotomy. The management of associated conditions, such as diabetes, obesity and hyperlipidemia, is especially important, given their association with more advanced liver disease. This may be achieved by optimizing medical treatment of these conditions, as well as through weight loss strategies.

EFFECT OF WEIGHT LOSS ON NAFLD

Because obesity is the most commonly associated condition with NAFLD, weight loss has traditionally been the most commonly suggested intervention. Patients are encouraged to lose weight through exercise and dietary fat restriction. Exercise is of great value as it reduces weight by preferentially decreasing visceral obesity while preventing the loss of muscle mass. It also enhances muscle insulin sensitivity even in the absence of weight loss. A number of studies suggest that NAFLD may improve after weight loss. Improvement in liver biochemistry and ultrasonographic appearance is a consistent finding with moderate weight reduction. However, serum aminotransferases are unreliable markers for follow-up, and do not provide accurate data on prognosis. Worsening of fibrosis can occur even as the levels of transaminases decline^[42]. A few studies have evaluated and shown histologic improvement.

The effects of weight reduction on hepatic tests and physical findings were studied in a retrospective review of thirty-nine obese patients without primary liver disease. A weight loss of >10% corrected abnormal liver tests, decreased hepatosplenomegaly and resolved some stigmata of liver disease^[43]. Early case series showed that improved liver chemistry and histology was evident with even a modest reduction in weight, as considerable extra weight persisted in the subjects under evaluation^[44,45].

Drenick *et al*^[46] studied liver biopsies of 41 obese patients undergoing massive weight reduction (>100 lb). Biopsies were obtained at various stages before, during and after weight loss. Based on the method of weight loss, patients were divided into three groups, including prolonged fasting, low-calorie dieting and intestinal bypass surgery. In the non-surgical groups, a transient increase in hepatocellular degeneration and focal necrosis was noted along with progressive diminution of fatty infiltration during weight loss. However, late biopsies revealed normal histology. In patients who underwent intestinal bypass, biopsies variously revealed massive fatty changes, cholestasis, polynuclear inflammatory infiltrates, diffuse fibrosis, bile-duct proliferation and fatal hepatic necrosis^[46]. These findings relating to jejunoileal bypass have been demonstrated in several studies^[47-49]. This surgical procedure has been abandoned in favor of safer weight-loss surgery.

In a study of twenty-five obese Japanese subjects^[50], fifteen underwent a program of restricted diet and exercise for a period of three months, while ten did not. Patients in the intervention group showed a significant decrease in BMI, aminotransferases, total protein, cholinesterase, total

cholesterol and fasting plasma glucose levels. In addition, steatosis was significantly improved on liver biopsy in these patients. The ten patients in the control group did not show any change in biochemical parameters or liver histology.

Regular body weight and biological measurements were obtained from 505 severely obese patients before and after undergoing gastroplasty^[24]. There was a high prevalence of biologic abnormalities associated with the metabolic syndrome at baseline. After a mean follow-up of 21 ± 14 mo post-surgery, significant reductions in biological markers of metabolic syndrome, such as blood glucose, insulin, and triglycerides, were noted. Also, total cholesterol, uric acid and fibrinogen and ALT levels were reduced, along with an increase in HDL cholesterol levels. Data on children with NASH, who underwent weight reduction, also showed improvement in biochemical and ultrasonographic findings^[51,52].

The effect of weight loss on NAFLD was studied in 36 obese patients. Paired liver biopsies were obtained, the first at the time of laparoscopic adjustable gastric band placement and the second after weight reduction. Initial biopsies showed steatosis alone in 12 patients and NASH in 23 patients. Initial fibrosis score of 2 or more was noted in 18 patients. Follow-up biopsies were obtained at 25.6 ± 10 mo after surgery. Weight loss resulted in a significant improvement in liver histology, with repeat biopsies showing NASH in only 4 patients and a fibrosis score of 2 or more in only 3 patients. Greater improvement was seen in patients who had been diagnosed with metabolic syndrome prior to surgery^[53].

Knobler *et al*^[54] suggested that NAFLD is not only associated with biochemical abnormalities of the metabolic syndrome, but it responds to their amelioration. Forty-eight patients with chronically elevated liver enzymes with clinical, ultrasound, and histologic findings consistent with fatty liver were evaluated. Most of the patients were overweight or obese (64%), 44% had diabetes mellitus, 29% had impaired glucose tolerance, and 17% were hyperinsulinemic. Dietary intervention was the primary mode of weight loss. This was supplemented by oral hypoglycemic or lipid-lowering drugs as needed. The results showed moderate weight loss (3.7 kg), improvement in fasting plasma glucose and lipid levels. An improvement in liver enzymes was noted in 96% patients with normalization in 50% patients.

In a recent pilot study, ten obese patients with NASH were treated with orlistat in addition to diet for weight reduction over a period of 6 mo^[55]. BMI, liver enzymes, hemoglobin A1c, fasting lipids, glucose and liver histology were assessed at baseline and at completion of the study. Mean weight loss was 22.7 lb. There was a significant decrease in the BMI, and levels of hemoglobin A1c, ALT, AST. Steatosis improved in six patients and fibrosis in three patients. Hickman *et al*^[56] found that modest weight loss through exercise and dietary intervention resulted in sustained improvements not only of ALT and fasting insulin levels, but also of health-related quality of life.

The above data demonstrates that NAFLD and NASH improve significantly with weight reduction. However, this must be done in a controlled manner over a period

of time, as rapid weight loss may lead to exacerbation of liver disease. This has been shown following drastic weight reduction through diet as well as through bariatric surgery. Forty-one patients who had weight loss on a very-low-calorie diet showed improvement in fatty change on liver biopsy. However, 24% developed portal inflammation or fibrosis. These were patients who underwent a very rapid weight reduction (>1.6 kg per week)^[57]. Liver biopsies obtained from patients undergoing gastroplasty for morbid obesity were compared before surgery and after weight loss (mean 32 ± 19 kg). There was a significant decrease in the prevalence of steatosis (38% of patients after weight loss *vs* 83% before). However, an increase in the prevalence of hepatitis was observed after significant weight reduction (26% of biopsies after weight loss *versus* 14% before)^[58]. The pathogenesis appears to be massive mobilization of fatty acids from visceral stores, which reach the liver via the portal vein and may be toxic to the liver. Therefore, initial target weight reduction should be 10% of baseline weight and should not exceed 1.6 kg/wk.

POPULAR DIETS

The effects of many popular diets on fatty liver are not known. However, metabolic improvements related to dietary weight reduction may favorably influence NASH. If dietary intervention can positively affect insulin resistance and other features of the metabolic syndrome, it would be important to know which particular diet is most beneficial. Conventional diets usually fall into two main categories. Those that alter the macronutrient composition of the diet and those that limit overall energy intake.

Alterations in macronutrient composition

Diets that promote weight loss by emphasizing their macronutrient make-up, as opposed to caloric intake, include low-fat and low-carbohydrate diets. Low-fat diets are traditionally the most recommended by medical professionals. Over time, these have been shown to be safe, cardio-protective and effective in weight loss. Adherence, however, has been a problem. Energy density of food is an important consideration. This refers to the energy (calories) in a given weight of food. Weight loss from a low-fat diet may be due to the low energy density of the diet. Both carbohydrates and proteins have an energy density of 4 kcal/kg, compared to 9 kcal/kg for fats. Researchers for the National Weight Control Registry found that members who maintained successful weight loss, consumed less energy and a lower percentage of energy from fat when compared to the general population^[59]. The low-fat (30%) diet is advocated by the National Cholesterol Education Program and by the American Heart Association.

Low carbohydrate diets have been popular periodically over the last several decades, and are currently undergoing a resurgence. An NPD survey on diet trends in the United States showed that in early 2004 about 9% of the population was on a low carbohydrate diet. These diets limit the composition and/or amount (<100 g/d) of carbohydrates, with an increase in dietary protein and

fat. They are marketed as low carbohydrate, high protein diets, although they could also be called low carbohydrate, high fat diets. A diet high in carbohydrates results in an increase in blood glucose, insulin and triglycerides, all of which are risk factors for the development of NAFLD. Carbohydrate restriction leads to ketosis resulting not only in weight loss, but also a decrease in blood glucose, insulin and triglyceride levels. Studies have shown these diets to be effective in short-term weight loss^[60,61]. Early weight loss is a result of diuresis associated with ketone and urea nitrogen excretion^[62]. However, over time, weight loss is a result of loss of body fat. Proponents believe that these diets have a high satiety level, which make them easier to adhere to. This is very important, as dietary adherence is one of the main challenges faced by dieters. Questions with regard to their nutritional adequacy and long-term effects have been raised. In the short-term, these diets have been found to be safe.

Popular low carbohydrate diets include the Atkins, South Beach and the Zone diets. Originally published by Dr. Robert Atkins in 1972 (Dr. Atkins diet revolution), the Atkins diet is the most popular low carbohydrate diet in the United States. Weight reduction is achieved in four phases. The first phase is an induction diet which limits carbohydrates to 20 g/d, but allows unlimited amounts of fat. In phase two, there is ongoing weight loss with easing of the carbohydrate restriction. Through phases three and four, pre-maintenance and maintenance, individuals determine the amount of carbohydrate they can consume while maintaining their weight loss^[63]. The South Beach diet consists of 3 phases, gradually increasing in proportion of carbohydrates and emphasizes good carbohydrates and fats. The Zone diet recommends a low carbohydrate, high protein diet, with macronutrient intake in the 40:30:30 ratio, i.e. 40% calories from carbohydrates, 30% from protein and 30% from fat.

Reduction in energy intake

Studies have found that weight loss on a calorie-restricted diet is due to decreased energy intake and not nutrient composition. Golay *et al*^[64] evaluated the effect of diets that were equally low in energy, but widely different in amounts of fat and carbohydrate over a 6-wk period. No significant difference in amount of weight loss was noted^[64]. Alford *et al*^[65] studied the effects of three different diets with a fixed caloric intake of 1 200 kcal/d. They found no significant difference in weight loss among diets with 25%, 45%, 75% carbohydrate. The authors concluded that weight loss is the result of reduction in caloric intake in proportion to caloric requirements. Schlundt *et al*^[66] found greater weight loss with a low-fat, calorie-restricted diet when compared to a low-fat, *ad libitum* carbohydrate intake diet. A systematic review of low carbohydrate diets by Freedman *et al*^[67] found weight loss to be associated with energy restriction and not carbohydrate restriction. It is likely that individuals on diets that alter macronutrient composition (low fat or low carbohydrate) actually lose weight because of a concurrent reduction in energy intake.

LOW-CARBOHYDRATE *VERSUS* LOW-FAT DIETS: EFFECTS ON BIOCHEMICAL MARKERS OF METABOLIC SYNDROME AND NAFLD

As mentioned previously, dietary weight reduction has been shown to have a positive effect on biochemical markers of the metabolic syndrome. This may translate into a positive effect on NAFLD. Encouraging histologic findings have been noted in these patients. Therefore, it is important to compare the various diets and determine which, if any, is most beneficial in NAFLD.

Low-carbohydrate diets have long been considered fad diets by the medical profession. Several questions have been raised regarding their weight loss potential and possible adverse effects. Public interest continues unabated, and books detailing low carbohydrate lifestyles are regulars on best seller lists. A number of studies have been published in recent years evaluating the effects of low-carbohydrate diets on weight loss as well as on metabolic markers, comparing these diets to traditional low-fat diets. However, none has compared their effects on liver histology and NAFLD. Effects on obesity and biomarkers of the metabolic syndrome will be reviewed, given their important etiologic association with NAFLD.

In an uncontrolled study, Westman *et al*^[68] showed that in mildly obese, motivated persons, a very low-carbohydrate diet led to sustained weight loss during a 6-mo period. Positive effects were also noted on the serum lipid profile, with a decrease in triglycerides, total and low-density lipoprotein cholesterol and an increase in high-density lipoprotein cholesterol levels. More recently, several randomized controlled trials comparing various diets have been published. Brehm *et al*^[69] studied the effects of a very low-carbohydrate diet on body composition and cardiovascular risk factors. Fifty three healthy, obese women were randomized to 6 mo of an *ad libitum* very low-carbohydrate diet *versus* a calorie-restricted low-fat diet. The very low carbohydrate group had more weight loss (8.5 ± 1.0 *vs* 3.9 ± 1.0 kg, $P < 0.001$) without any deleterious effect on cardiovascular risk factors. Foster *et al* randomized 63 non-diabetic obese subjects to a low carbohydrate (Atkins) *versus* a conventional diet (low fat, high carbohydrate, calorie-restricted diet). The Atkins group had more weight loss at 3 mo (-6.8 ± 5.0 % *vs* -2.7 ± 3.7 %) and 6 mo (-7.0 ± 6.5 % *vs* -3.2 ± 5.6 %), but at 12 mo the difference between the 2 groups was not significant (-4.4 ± 6.7 % *vs* -2.5 ± 6.3 %). The low carbohydrate group was associated with a greater decrease in triglycerides and a greater increase in high-density lipoprotein cholesterol levels. No difference in the total of low-density lipoprotein cholesterol levels was seen between the both groups. Insulin sensitivity increased in both groups without any significant difference between groups^[61]. In a study by Samaha *et al*^[60], 132 severely obese subjects with a mean body mass index (BMI) of 43 (diabetes mellitus in 39% and metabolic syndrome in 43%) were randomized to a low-carbohydrate or a low-fat, calorie-restricted diet. At 6 mo, the low carbohydrate group had more weight loss (-5.8 ± 8.6 kg *vs* -1.9 ± 4.2 kg

and a greater decrease in triglyceride levels (-20 ± 43 % *vs* -4 ± 31 %). In non-diabetics, the insulin sensitivity improved more, and in diabetics, fasting glucose levels decreased more in the low carbohydrate group^[60]. No adverse effects on the serum lipid levels were observed. The 12-mo data from this study was published in 2004. Weight loss between the two groups at this point was no longer significant. The favorable metabolic response to the low-carbohydrate diet, however, persisted^[70]. A recently published study of 120 overweight, hyperlipidemic subjects showed similar findings. At 24 wk, the low carbohydrate (Atkins) group had greater weight loss (mean -12.9 % *vs* -6.7 %, $P < 0.001$). Effects on triglyceride and high-density lipoprotein cholesterol levels were also more favorable with the low-carbohydrate diet^[71]. Comparison of the National Cholesterol Education Program (NCEP) diet with a modified low-carbohydrate diet (low in total carbohydrates but higher in complex carbohydrates, protein and monounsaturated fat) showed a significantly greater weight loss in the modified low-carbohydrate diet over a period of 12 wk. This study did not show any significant difference between groups in blood lipid levels^[72]. A low-carbohydrate diet over 12 wk was also found to be more effective than a low-fat diet for weight loss in overweight adolescents without adversely affecting their lipid profile^[73]. In comparing 4 popular diets (Atkins, Zone, Weight Watcher's and Ornish) over one year, all were found to reduce weight modestly, and to have a significant reduction in low-density lipoprotein/high-density lipoprotein cholesterol ratio. No significant difference was noted between diets, and no diet-related adverse effects were noted^[74]. Evaluation of 3 different diets in overweight, insulin-resistant women showed greater weight reduction from the Atkins and Zone diets when compared to a conventional low-fat diet. A greater reduction in triglycerides and waist circumference was also noted with these diets. A significant increase in low-density lipoprotein levels was noted in 25% of subjects on the Atkins diet, whereas this was seen in only 13% of subjects on the low-fat diet and 3% of those on the Zone diet^[75]. Improvement in characteristics of the metabolic syndrome as demonstrated by a decrease in triglycerides, triglyceride/high-density lipoprotein ratio, postprandial lipemia and increase in low-density lipoprotein particle size was shown in overweight men on a very low-carbohydrate diet^[76]. Similarly, improved insulin sensitivity and prevention of HDL cholesterol decline were noted in overweight men^[77].

Results from these studies suggest that a low-carbohydrate diet results in weight loss and may even be more effective than a conventional, low-fat diet in the short-term period. Greater weight loss may be the result of the monotony and simplicity of the diet inhibiting appetite and food intake^[78]. Enhanced satiety, palatability and novelty of the diet may also play a role^[60,70]. There has been great concern regarding negative effects on renal function, bone health and cancer risk. Some studies showed that low-carbohydrate diets had a higher incidence of minor side-effects, such as constipation, headache, halitosis, muscle cramps, diarrhea, rash and general weakness^[71]. The major concern of an adverse effect on serum lipids, renal and cardiovascular health was not realized in these

studies. Again, these studies have relatively short follow-up periods, and the effect on low-density lipoprotein cholesterol levels remains uncertain, and requires further study. Caution must be exercised in subjects with baseline abnormal low-density lipoprotein cholesterol levels. Effects on biochemical markers associated with the metabolic syndrome appear to be more favorable with low-carbohydrate diets. In general, these diets show greater improvements in insulin sensitivity, triglyceride and high-density cholesterol levels. It is possible that for patients with the metabolic syndrome, a low-carbohydrate diet may be more advantageous. This, in turn, may positively affect NAFLD.

CONCLUSION

As the prevalence of obesity increases, so will that of the metabolic syndrome and NAFLD. It is now recognized that the consequences of NAFLD are not always benign. While pure steatosis alone is generally an indolent disease, steatohepatitis can be a progressive disease leading to cirrhosis and even liver failure. The etiologic association between NAFLD and the metabolic syndrome is so well established that NAFLD is considered a hepatic manifestation of the disease. Obesity and insulin resistance are associated with more histologically advanced disease. Given this scenario, it is important to develop new strategies to treat and prevent NASH. While it is known that dietary weight loss improves markers of the metabolic syndrome and data also suggest that judicious weight loss affects the liver favorably in NAFLD, the best dietary approach is yet unknown. Traditionally, a low-fat diet has been recommended, but recent studies, show greater short-term weight loss and greater improvement in markers of the metabolic syndrome without significant adverse effects with low-carbohydrate diets. This raises the question of whether low-carbohydrate diets should be recommended as part of a weight loss strategy for our patients. At this point, questions regarding the nutritional adequacy and long-term safety remain. While studies have evaluated the effect of these diets on weight loss, cardiovascular and metabolic marker studies are needed to evaluate the effect of these diets specifically on NAFLD.

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REVIEW

Epidemiology of gastric cancer

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Abstract

The incidence and mortality of gastric cancer have fallen dramatically in US and elsewhere over the past several decades. Nonetheless, gastric cancer remains a major public health issue as the fourth most common cancer and the second leading cause of cancer death worldwide. Demographic trends differ by tumor location and histology. While there has been a marked decline in distal, intestinal type gastric cancers, the incidence of proximal, diffuse type adenocarcinomas of the gastric cardia has been increasing, particularly in the Western countries. Incidence by tumor sub-site also varies widely based on geographic location, race, and socio-economic status. Distal gastric cancer predominates in developing countries, among blacks, and in lower socio-economic groups, whereas proximal tumors are more common in developed countries, among whites, and in higher socio-economic classes. Diverging trends in the incidence of gastric cancer by tumor location suggest that they may represent two diseases with different etiologies. The main risk factors for distal gastric cancer include *Helicobacter pylori* (*H pylori*) infection and dietary factors, whereas gastroesophageal reflux disease and obesity play important roles in the development of proximal stomach cancer. The purpose of this review is to examine the epidemiology and risk factors of gastric cancer, and to discuss strategies for primary prevention.

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Key words: Epidemiology; Gastric cancer

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INTRODUCTION

Overall, gastric cancer incidence and mortality have

fallen dramatically over the past 70 years^[1]. Despite its recent decline, gastric cancer is the fourth most common cancer and the second leading cause of cancer-related death worldwide^[2,3]. In 2000, about 880 000 people were diagnosed with gastric cancer and approximately 650 000 died of the disease^[4].

The two main tumor sites of gastric adenocarcinoma are proximal (cardia) and distal (noncardia). Despite a decline in distal gastric cancers, proximal tumors have been increasing in incidence since the 1970s, especially among males in the Western countries^[5,6]. These gastric tumor types predominate in populations from different geographic locations, racial and socio-economic groups. They may also differ in genetic susceptibility, pathologic profile, clinical presentation, and prognosis. The observed differences between gastric cancers by anatomic site suggest that they are distinct diseases with different etiologies. Detailed epidemiological analyses of their demographic trends and risk factors will help guide future cancer control strategies.

PATHOLOGIC CONSIDERATIONS

About 90% of stomach tumors are adenocarcinomas, which are subdivided into two main histologic types: (1) well-differentiated or intestinal type, and (2) undifferentiated or diffuse type. The intestinal type is related to corpus-dominant gastritis with gastric atrophy and intestinal metaplasia, whereas the diffuse type usually originates in pangastritis without atrophy.

The intestinal type is more common in males, blacks, and older age groups, whereas the diffuse type has a more equal male-to-female ratio and is more frequent in younger individuals^[7,8]. Intestinal type tumors predominate in high-risk geographic areas, such as East Asia, Eastern Europe, Central and South America, and account for much of the international variation of gastric cancer^[9]. Diffuse type adenocarcinomas of the stomach have a more uniform geographic distribution^[10]. A decline in the incidence of the intestinal type tumors in the corpus of the stomach accounts for most of the recent decrease in gastric cancer rates worldwide^[11]. In contrast, the incidence of diffuse type gastric carcinoma, particularly the signet ring type, has been increasing^[12].

DEMOGRAPHIC TRENDS

Time trends

In the 1930s, gastric cancer was the most common cause of cancer death in US and Europe. During the past 70 years, mortality rates have fallen dramatically

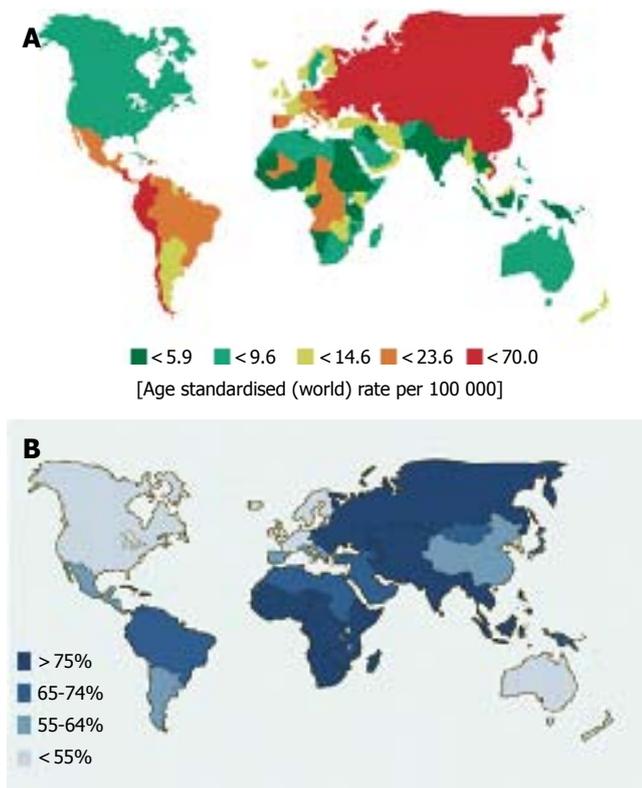


Figure 1 (A) Incidence of stomach cancer in males. (B) Prevalence of *H. pylori* infection in asymptomatic adults. (Data adopted from Parkin *et al.*^[6])

in all developed countries largely due to unplanned prevention. However, in the past 30 years, the incidence of gastric cardia adenocarcinoma rose by five- to six-fold in developed countries^[13-19]. Gastric cardia tumors now account for nearly half of all stomach cancers among men from US and UK^[6,20]. There has also been a rising trend in esophageal adenocarcinoma, in which obesity, gastroesophageal reflux disease (GERD), and Barrett's esophagus are major etiologic factors. Gastric cardia cancers share certain epidemiologic features with adenocarcinomas of the distal esophagus and gastroesophageal (GE) junction, suggesting that they represent a similar disease entity.

Geographic variation

Gastric cancer incidence rates vary by up to ten-fold throughout the world. Nearly two-thirds of stomach cancers occur in developing countries^[4]. Japan and Korea have the highest gastric cancer rates in the world^[21,22]. High-incidence areas for noncardia gastric adenocarcinoma include East Asia, Eastern Europe, and Central and South America^[20,23]. Low incidence rates are found in South Asia, North and East Africa, North America, Australia, and New Zealand (Figure 1A).

In Japan, gastric cancer remains the most common type of cancer among both men and women. Age-standardized incidence rates in Japan are 69.2 per 100 000 in men and 28.6 per 100 000 in women^[3]. In contrast to the increasing incidence of proximal tumors in the West, distal tumors continue to predominate in Japan. However, even in Japan, the proportion of proximal stomach cancers has increased among men^[24].

Migrant populations from high-risk areas such as Japan show a marked reduction in risk when they move to low-incidence regions such as the US^[25]. Subsequent generations acquire risk levels approximating those of the host country^[20,23].

Sex, race, and age distribution

Noncardia gastric cancer has a male-to-female ratio of approximately 2:1^[20,23]. Incidence rates are significantly higher among blacks and lower socio-economic groups, and in developing countries^[20]. Incidence rises progressively with age, with a peak incidence between 50 and 70 years.

In contrast, for gastric cardia carcinomas, men are affected five times more than women and whites twice as much as blacks^[26]. In addition, the incidence rates of proximal gastric cancers are relatively higher in the professional classes^[27]. Different rates of genetic polymorphisms according to tumor sub-site suggest variation in susceptibility to stomach cancer by tumor location^[28]. These findings suggest that noncardia and cardia adenocarcinomas are distinct biological entities.

SURVIVAL

For the past few decades, gastric cancer mortality has decreased markedly in most areas of the world^[29,30]. However, gastric cancer remains a disease of poor prognosis and high mortality, second only to lung cancer as the leading cause of cancer-related death worldwide. In general, countries with higher incidence rates of gastric cancer show better survival rates than countries with lower incidence^[31]. This association is largely due to a difference in survival rates based on tumor location within the stomach. Tumors located in the gastric cardia have a much poorer prognosis compared to those in the pyloric antrum, with lower 5-year survival and higher operative mortality^[32].

In addition, the availability of screening for early detection in high-risk areas has led to a decrease in mortality. In Japan where mass screening programs are in place, mortality rates for gastric cancer in men have more than halved since the early 1970s^[33]. When disease is confined to the inner lining of the stomach wall, 5-year survival is on the order of 95%. In contrast, few gastric cancers are discovered at an early stage in US, leading to 5-year relative survival rates of less than 20%^[34]. Similarly in European countries, the 5-year relative survival rates for gastric cancer vary from 10% to 20%^[35,36]. Host-related factors may also affect prognosis, as a US study demonstrated that gastric cancers in persons of Asian descent had a better prognosis compared to non-Asians^[37].

RISK FACTORS

Gastric cancer is a multifactorial disease. The marked geographic variation, time trends, and the migratory effect on gastric cancer incidence suggest that environmental or lifestyle factors are major contributors to the etiology of this disease.

Helicobacter pylori infection

H. pylori is a gram-negative bacillus that colonizes the stomach and may be the most common chronic bacterial

infection worldwide^[38]. Countries with high gastric cancer rates typically have a high prevalence of *H pylori* infection, and the decline in *H pylori* prevalence in developed countries parallels the decreasing incidence of gastric cancer^[39,40] (Figure 1B). In US, the prevalence of *H pylori* infection is <20% at the age 20 years and 50% at 50 years^[41]. In Japan, it is also <20% at 20 years, but increases to 80% over the age of 40 years^[42] and in Korea, 90% of asymptomatic adults over the age of 20 years are infected by *H pylori*^[43]. The increase in prevalence with age is largely due to a birth cohort effect rather than late acquisition of infection. *H pylori* infection is mainly acquired during early childhood, likely through oral ingestion, and infection persists throughout life^[44]. Prevalence is closely linked to socio-economic factors, such as low income and poor education, and living conditions during childhood, such as poor sanitation and overcrowding^[45-49].

The association between chronic *H pylori* infection and the development of gastric cancer is well established^[50-53]. In 1994, the International Agency for Research on Cancer classified *H pylori* as a type I (definite) carcinogen in human beings^[54]. In Correa's model of gastric carcinogenesis, *H pylori* infection triggers the progressive sequence of gastric lesions from chronic gastritis, gastric atrophy, intestinal metaplasia, dysplasia, and finally, gastric adenocarcinoma^[55]. Several case-control studies have shown significant associations between *H pylori* seropositivity and gastric cancer risk, with about a 2.1- to 16.7-fold greater risk compared to seronegative individuals^[56-62]. Prospective studies have also supported the association between *H pylori* infection and gastric cancer risk^[50-52,63]. Perhaps the most compelling evidence for the link between *H pylori* and gastric cancer comes from a prospective study of 1 526 Japanese participants in which gastric cancers developed in 2.9% of infected people and in none of the uninfected individuals^[64]. Interestingly, gastric carcinomas were detected in 4.7% of *H pylori*-infected individuals with non-ulcer dyspepsia.

The vast majority of *H pylori*-infected individuals remain asymptomatic without any clinical sequelae. Cofactors, which determine that *H pylori*-infected people are at particular risk for gastric cancer, include bacterial virulence factors and proinflammatory host factors. Gastric cancer risk is enhanced by infection with a more virulent strain of *H pylori* carrying the cytotoxin-associated gene A (*cagA*)^[65,66]. Compared to *cagA*- strains, infection by *H pylori cagA+* strains was associated with an increased risk of severe atrophic gastritis and distal gastric cancer^[67-70]. In the Western countries, about 60% of *H pylori* isolates are *cagA+*^[71], whereas in Japan, nearly 100% of the strains possess functional *cagA*^[72,73]. Host factors associated with an increased risk of gastric cancer include genetic polymorphisms which lead to high-level of expression of the proinflammatory cytokine, interleukin-1 β ^[74,75].

The effects of *H pylori* on gastric tumor development may vary by anatomical site. The falling incidence of *H pylori* infection and noncardia gastric cancer in developed countries has been diametrically opposed to the rapid increase in the incidence of gastric cardia adenocarcinoma^[76]. Based on a meta-analysis of prospective cohort studies, *H pylori* infection was associated

with the risk of noncardia gastric cancer, but not cardia cancer^[77]. Other studies demonstrated a significant inverse association between *H pylori* infection, particularly *cagA+* strains, and the development of gastric cardia and esophageal adenocarcinomas^[78,79]. In the Western countries, where the prevalence of *H pylori* infection is falling, GERD and its sequelae are increasing. Studies have shown that severe atrophic gastritis and reduced acid production associated with *H pylori* infection significantly reduced the risk of GERD^[80-83]. However, recent studies have found conflicting results on whether *H pylori* eradication therapy increases the risk of esophagitis and gastric cardia adenocarcinoma^[84-91]. Thus, the protective effect of *H pylori* against cardia tumors remains controversial.

Dietary factors

It is unlikely that *H pylori* infection alone is responsible for the development of gastric cancer. Rather, *H pylori* may produce an environment conducive to carcinogenesis and interact with other lifestyle and environmental exposures. There is evidence that consumption of salty foods and *N*-nitroso compounds and low intake of fresh fruits and vegetables increases the risk of gastric cancer. *H pylori* gastritis facilitates the growth of nitrosating bacteria, which catalyze the production of carcinogenic *N*-nitroso compounds^[92]. In addition, *H pylori* infection is known to inhibit gastric secretion of ascorbic acid, which is an important scavenger of *N*-nitroso compounds and oxygen free radicals^[93].

Salt-preserved foods and dietary nitrite found in preserved meats are potentially carcinogenic. Intake of salted food may increase the risk of *H pylori* infection and act synergistically to promote the development of gastric cancer. In animal models, ingestion of salt is known to cause gastritis and enhance the effects of gastric carcinogens^[94,95]. Mucosal damage induced by salt may increase the possibility of persistent infection with *H pylori*^[96]. Several case-control studies have shown that a high intake of salt and salt-preserved food was associated with gastric cancer risk^[97-103], but evidence from prospective studies is inconsistent^[104-107]. *N*-nitroso compounds are carcinogenic in animal models and are formed in the human stomach from dietary nitrite. However, case-control studies have shown a weak, nonsignificant increased risk of gastric cancer for high vs low nitrite intake^[97,108-110]. Prospective studies have reported significant reductions in gastric cancer risk arising from fruit and vegetable consumption^[111-114]. The worldwide decline in gastric cancer incidence may be attributable to the advent of refrigeration, which led to decreased consumption of preserved foods and increased intake of fresh fruits and vegetables.

Animal studies have shown that polyphenols in green tea have antitumor and anti-inflammatory effects. In preclinical studies, polyphenols have antioxidant activities and the ability to inhibit nitrosation, which have been implicated as etiologic factors of gastric cancer^[115-117]. Although various case-control studies have shown a reduced risk of gastric cancer in relation to green tea consumption^[118-121], recent prospective cohort studies found no protective effect of green tea on gastric cancer risk^[122-125].

Table 1 Epidemiologic differences between cardia and noncardia gastric cancer

| | Cardia | Noncardia |
|---------------------------|------------|------------|
| | Increasing | Decreasing |
| Incidence | | |
| Geographic location | | |
| Western countries | + | - |
| East Asia | - | + |
| Developing countries | - | + |
| Age | ++ | ++ |
| Male gender | ++ | + |
| Caucasian race | + | - |
| Low socio-economic status | - | + |
| <i>H pylori</i> infection | ? | + |
| Diet | | |
| Preserved foods | + | + |
| Fruits/vegetables | - | - |
| Obesity | + | ? |
| Tobacco | + | + |

NOTE: ++, strong positive association; +, positive association; -, negative association; ?, ambiguous studies.

Tobacco

Prospective studies have demonstrated a significant dose-dependent relationship between smoking and gastric cancer risk^[126,127]. The effect of smoking was more pronounced for distal gastric cancer, with adjusted rate ratios of 2.0 (95% CI, 1.1-3.7) and 2.1 (95% CI, 1.2-3.6) for past and current smokers, respectively^[128]. There is little support for an association between alcohol and gastric cancer^[129].

Obesity

Obesity is one of the main risk factors for gastric cardia adenocarcinoma^[130,131]. Obesity can promote GE reflux disease which predisposes to Barrett's esophagus, a metaplastic precursor state for adenocarcinoma of the esophagus and GE junction^[132,133]. A Swedish study found that the heaviest quarter of the population had a 2.3-fold increased risk for gastric cardia adenocarcinoma compared to the lightest quartile of the population^[134]. A recent prospective study from US found that body mass index was significantly associated with higher rates of stomach cancer mortality among men^[135]. Thus, risk factors positively associated with adenocarcinoma of the esophagus and gastric cardia include obesity, GE reflux, and the presence of Barrett's esophagus. A summary of the main differences between cardia and noncardia gastric cancer can be found in Table 1.

Other

Less common risk factors for gastric cancer include radiation^[136], pernicious anemia^[137], blood type A^[138], prior gastric surgery for benign conditions^[139], and Epstein-Barr virus^[140-142]. In addition, a positive family history is a significant risk factor, particularly with genetic syndromes such as hereditary nonpolyposis colon cancer and Li-Fraumeni syndrome^[143-145].

PREVENTION OF GASTRIC CANCER

Lifestyle modifications

Because gastric cancer is often associated with a poor

prognosis, the main strategy for improving clinical outcomes is through primary prevention. Reduction in gastric cancer mortality is largely due to unplanned prevention. The widespread introduction of refrigeration has led to a decrease in the intake of chemically preserved foods and increased consumption of fresh fruits and vegetables^[98,146]. A decline in the prevalence of *H pylori* infection may be due to improvements in sanitary and housing conditions, as well as the use of eradication therapy^[54]. In addition, reduced tobacco smoking at least in males may have contributed to the decline in gastric cancer incidence^[147]. Therefore, modifiable risk factors, such as high salt and nitrite consumption, low fruit and vegetable intake, cigarette smoking, and *H pylori* infection, may be targeted for prevention.

Helicobacter pylori eradication

Public health measures to improve sanitation and housing conditions are the key factors in reducing the worldwide prevalence of *H pylori* infection. *H pylori* eradication therapy is another potential strategy for gastric cancer chemoprevention. A 7 to 14 d course of two antibiotics and an antisecretory agent has a cure rate of about 80% with durable responses^[148]. However, higher reinfection rates are seen in developing countries after people have had effective eradication therapy^[149]. In Japanese patients treated for early gastric cancer, *H pylori* eradication therapy resulted in a significantly lower rate of gastric cancer recurrence^[150]. A randomized controlled chemoprevention trial showed that antimicrobial therapy directed against *H pylori* or dietary supplementation with antioxidants increased the regression rate of gastric atrophy and intestinal metaplasia compared to placebo^[151]. In a randomized, placebo-controlled primary prevention trial conducted in a high-risk region of China, 1 630 healthy carriers of *H pylori* infection were randomized to a 2-wk course of eradication treatment or placebo^[152]. Although the incidence of gastric cancer was similar in both groups after 7.5 years of follow-up, post hoc analysis of a subgroup of *H pylori* carriers without precancerous lesions at baseline showed a significant decrease in the development of gastric cancer with eradication therapy.

Several large-scale chemoprevention trials of *H pylori* eradication therapy with gastric cancer endpoints are ongoing. Potential downsides of widespread eradication therapy in asymptomatic carriers include developing antibiotic-resistant strains of *H pylori* and perhaps increasing the risk of GERD and adenocarcinoma of the esophagus and gastric cardia.

Antioxidants

High intake of antioxidants, such as vitamins C and E and β -carotene, may have a protective effect on the risk of gastric cancer. High serum levels of α -carotene, β -carotene, lycopene, and vitamin C were significantly associated with reduced risk of gastric cancer in a cohort from Shanghai, China^[153]. A randomized trial in Linxian, China showed a reduced risk of both cardia and noncardia gastric cancers in individuals supplemented with a combination of selenium, β -carotene, and α -tocopherol^[154]. However, a randomized trial from

Finland showed no association between α -tocopherol or β -carotene supplementation and the prevalence of gastric cancer in elderly men with atrophic gastritis^[155]. Another prospective study from the US Cancer Prevention Study II cohort found that vitamin supplementation did not significantly reduce the risk of stomach cancer mortality^[156]. Therefore, dietary supplementation may only play a preventive role in populations with high rates of gastric cancer and low intake of micronutrients.

COX-2 inhibitors

Cyclooxygenase-2 (COX-2) plays a role in cell proliferation, apoptosis, and angiogenesis, and may be involved in gastric carcinogenesis^[157,158]. Increasing levels of COX-2 are present in the progression from atrophic gastritis to intestinal metaplasia and adenocarcinoma of the stomach^[159]. Exposure to cigarette smoke, acidic conditions, and *H pylori* infection all induce COX-2 expression^[160-162]. Furthermore, McCarthy *et al.* showed that COX-2 expression in the antral mucosa was reduced in the epithelium after successful eradication of *H pylori*^[163].

Aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) are thought to inhibit cancer cell growth primarily through the inhibition of COX-2, and evidence is mounting that COX-2 inhibitors may be beneficial in preventing upper gastrointestinal malignancies. Compared to colorectal cancer, the association between NSAID use and the development of gastric cancer has been studied less extensively^[164-166]. A recent meta-analysis showed that NSAID use was associated with a reduced risk of noncardia gastric adenocarcinoma^[167]. Thus, COX-2 inhibitors may provide a chemopreventive strategy against gastric carcinogenesis.

Endoscopic screening and surveillance

Because of the high risk of gastric cancer in Japan, there has been a national endoscopic surveillance program within the commercial workforce. Annual screening with a double-contrast barium technique and endoscopy is recommended for persons over the age of 40 years^[168]. With mass screening, about half of gastric tumors are being detected at an early stage in asymptomatic individuals and the mortality rate from gastric cancer has more than halved since the early 1970s^[33]. An intervention study in China is underway which involves a comprehensive approach to gastric cancer prevention, including *H pylori* eradication, nutritional supplements, and aggressive screening with double contrast X-ray and endoscopic examination. In the first four years after intervention, the relative risk of overall mortality with this intervention for a high-risk group was 0.51 (95% CI, 0.35-0.74)^[169]. This study suggests that targeting high-risk populations for aggressive screening and prevention may decrease gastric cancer mortality.

CONCLUSION

In summary, cardia and noncardia gastric cancers exhibit unique epidemiologic features characterized by marked geographic variation, diverging time trends, and differences based on race, sex, and socio-economic status. *H pylori*

infection and dietary factors appear to be the main causative agents for distal gastric cancer, whereas GERD and obesity play a primary role in proximal gastric cancer. Future directions in primary prevention should target modifiable risk factors in high-risk populations. In the planning and evaluation of gastric cancer control activities, detailed demographic analyses will inform future screening and intervention studies.

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Stem cells and cancer: Evidence for bone marrow stem cells in epithelial cancers

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Abstract

Cancer commonly arises at the sites of chronic inflammation and infection. Although this association has long been recognized, the reason has remained unclear. Within the gastrointestinal tract, there are many examples of inflammatory conditions associated with cancer, and these include reflux disease and Barrett's adenocarcinoma of the esophagus, Helicobacter infection and gastric cancer, inflammatory bowel disease and colorectal cancer and viral hepatitis leading to hepatocellular carcinoma. There are several mechanisms by which chronic inflammation has been postulated to lead to cancer which includes enhanced proliferation in an endless attempt to heal damage, the presence of a persistent inflammatory environment creating a pro-carcinogenic environment and more recently a role for engraftment of circulating marrow-derived stem cells which may contribute to the stromal components of the tumor as well as the tumor mass itself. Here we review the recent advances in our understanding of the contributions of circulating bone marrow-derived stem cells to the formation of tumors in animal models as well as in human beings.

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INTRODUCTION

The association between cancer and inflammation has been recognized for over 2 000 years^[1,2]. Virchow recognized that many tumors arise in a setting of chronic inflammation, and later, Dvorak aptly described cancer as "wounds that do not heal"^[3]. It is estimated that up to 15% of cancers worldwide are associated with chronic infection^[4] and there are many more examples throughout the body of cancer associated with inflammation of unclear etiology. Within the GI tract, examples include esophageal adenocarcinoma arising in the setting of Barrett's metaplasia, gastric cancer occurring secondary to Helicobacter infection, colorectal cancer occurring in longstanding inflammatory bowel disease and hepatocellular carcinoma secondary to viral hepatitis (Table 1). There are several mechanisms by which inflammation and a cycle of chronic injury/repair has been postulated to lead to cancer, including the push for continued proliferation, an abnormal inflamed-stromal environment, and more recently we recognize a role for engraftment of circulating marrow-derived stem cells, which may contribute to the stromal components of the tumor as well as the epithelial component of the tumor mass itself.

INFLAMMATION: THE RELATIONSHIP BETWEEN TISSUE INJURY, REPAIR AND CANCER

Proliferation of cells alone is not sufficient to cause cancer, rather proliferation in the setting of altered growth signals from inflammatory cell infiltrates and DNA damaging agents released from infiltrating leukocytes promote malignant degeneration. Wound healing, which requires subversion of usual growth programs, mobilization and migration of cells and a heightened resistance to apoptosis, embodies all the properties of malignant cells with one exception, that is, healing is self limited. Successful wound healing results in restoration of tissue integrity and resolution of inflammation, reinstating homeostatic growth control. The tissue environments of longstanding unrelenting chronic infection or idiopathic chronic inflammation are persistent states of inadequate wound healing. Within this setting, inflammatory cells produce highly reactive oxygen and nitrogen species, which interact with cellular DNA inducing point mutations, deletions or rearrangements. Usually DNA damage such as this triggers

Table 1 Infection and inflammation associated with cancers

| Cause | Site |
|---|---|
| Chewing tobacco/oral irritation | Oral cancers |
| Smoking/chronic bronchitis | Lung |
| Asbestos | Mesothelioma |
| Reflux disease | Barretts' adenocarcinoma of the esophagus |
| Chronic Helicobacter infection | Gastric adenocarcinoma and lymphoma |
| Chronic pancreatitis | Pancreatic cancer |
| Opisthorchis sinensis infection (liver fluke) | Cholangiocarcinoma |
| Viral hepatitis | Hepatocellular carcinoma |
| Ulcerative colitis and Crohn's disease | Colorectal carcinoma |
| Human papilloma virus | Anogenital carcinomas |
| Schistosomiasis | Bladder cancer |
| Pelvic inflammatory disease | Ovarian cancer |
| Chronic osteomyelitis | Osteosarcoma |
| Chronic scar tissue | "Scar" cancer arising in the lung, skin and other areas of scarring |

apoptosis. However, in areas of chronic inflammation and repair, growth programs are corrupted and the environment is poised to allow replication and survival of cells, which under normal situations would either be quiescent or lost to apoptosis. Allowing cells to continually divide in an environment conducive to DNA damage may result in the accumulation of genetic defects and the emergence of malignant cells.

Which cell is the target of malignant transformation has been the area of much debate. Original thought was that a terminally differentiated cell would acquire enough genetic damage to replicate endlessly. This would require multiple genetic alterations in key cell signaling cascades to allow autonomous growth. A more likely scenario, however, is that these cells would undergo apoptosis or be sloughed off as a normal part of organ turnover prior to "backing up" the differentiation ladder sufficiently to acquire independent growth. More recently, focus has been on tissue-derived stem cells as the source of cancer. Tissue stem cells possess several important features making them attractive candidates for malignant degeneration. These include long life span, relative apoptosis resistance and ability to replicate for extended periods of time. In the setting of chronic inflammation, progenitor or stem cells within the peripheral tissue are forced to undergo multiple rounds of cell division predisposing to the accumulation of mutations. While restricted progenitors or even differentiated cells can still become transformed, in most cases, it has been believed that early stem cells are the targets of transformation.

If one looks at areas of high proliferative capacity (high BrdU or PCNA staining in tissue) as the stem cell zone, then this theory of peripheral stem cells as the cells of origin of cancer must be reconciled with the fact that this supposed stem cell compartment is often the most damaged and depleted by agents thought to be carcinogens^[5]. With chronic inflammation leading to atrophic changes attributed to peripheral stem cell exhaustion, the very cell thought to be transformed is lost - leading us to investigate if an alternate stem cell compartment is responsible for peripheral cancers arising in the setting of inflammation. In order to understand this concept, and why the bone marrow-derived stem cell (BMDC) is a "logical choice"

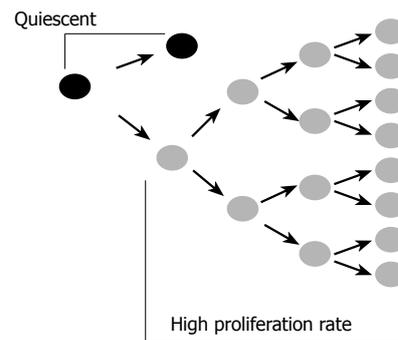


Figure 1 A proposed model for the cancer stem cell. The cancer stem cell replicates itself asymmetrically, thus maintaining one daughter stem cell identical to itself. This remains in a relatively quiescent state. The asymmetric division also produces another daughter cell with a high proliferative rate which rapidly divides to sustain the tumor mass.

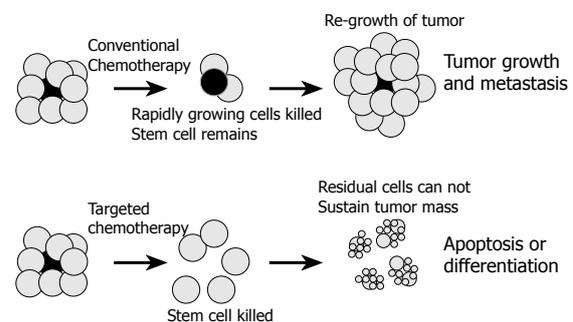


Figure 2 Conventional versus stem cell-targeted chemotherapy. Conventional chemotherapy and radiotherapy targets rapidly dividing cells, and may shrink tumor mass substantially. However, the stem cell (gray), which is relatively quiescent, is not affected. Regrowth of tumor from surviving stem cell leads to regrowth of tumor and treatment failure. Chemotherapy targeted at the stem cell would remove the source of new cell growth, and allow residual cells within the tumor to be targeted with chemotherapy, differentiating agents or therapy aimed at inducing apoptosis, thus successfully eliminating the tumor.

for a cancer stem cell, we need to understand a few points about cancer stem cells, and BMDCs in general.

Stem cells in cancer - cancer as an abnormal organ

A tumor mass can be compared to an abnormal organ; in that it is composed of a heterogeneous mixture of cell types and of cells possessing different proliferative capacities and different levels of differentiation. Tumor cells are admixed with fibroblasts, endothelial cells and inflammatory cells comprising the tumor stroma. This tumor stroma is being increasingly recognized as a critical contributor to malignant growth and survival of the tumor mass; however, stromal cells themselves are usually not malignant. Likewise, not all cells within a tumor are malignant - meaning not all cells can form tumors if transplanted at another site, or into a secondary host. In fact, the majority of cells within a tumor are incapable of independent growth and are readily susceptible to apoptosis. Only a small fraction of cells within a tumor are capable of independent growth, and fulfill the criteria described for cancer stem cells^[6-8]. These cells have metastatic potential, form tumors in secondary hosts and are believed to be responsible for continual renewal of cells within the tumor mass. These cells are likely to proliferate slowly and asymmetrically,

self renewing the stem cell population and giving rise to daughter cells, which proliferate to sustain tumor growth (Figure 1). Conventional anti-cancer chemotherapy and radiotherapy target rapidly dividing daughter cells, affecting the bulk of the tumor mass, but leave the cancer stem cell intact, explaining the often rapid recurrence of tumor bulk, once therapy is stopped (Figure 2). At present, the most pressing issue for cancer research is to identify the cancer stem cell and exploit its unique characteristics with targeted therapies.

Bone marrow stem cells

BMDCs are a heterogeneous group of cells isolated from the bone marrow which are capable of repopulating the hematopoietic system of a lethally irradiated immunologically compatible secondary host. These cells have been divided into at least two main categories; the hematopoietic and mesenchymal stem cells (MSC). Hematopoietic stem cells are traditionally regarded as the cells which give rise to the formed elements of the blood and have been used extensively in human bone marrow transplantation. Thus, hematopoietic stem cells have been extensively studied and defined with regard to surface markers, growth characteristics and repopulation potential^[9]. Less well defined are the MSC. This term MSC, as defined in the literature, is the heterogeneous population of cells isolated as the adherent population, when total marrow is placed in culture^[10]. These cells give rise to adipocyte, chondrocyte, cells of osteocyte lineages and the marrow mesenchyma, which is vital for optimal hematopoiesis^[11-13].

Work from multiple laboratories demonstrates surprising roles for marrow-derived stem cells in addition to hematopoiesis, stressing that the potential for differentiation may be much greater than originally believed. Markers defining cell subpopulations within the marrow are not standardized in these studies, making direct comparison of data between laboratories challenging; however, one thing remains consistent - cells within the bone marrow have a markedly greater differentiation potential than originally believed. For the purposes of this discussion, the term BMDCs will be broadly used to refer to cells derived from the marrow, and will encompass hematopoietic stem cells, MSC multipotent progenitor cells and whole marrow.

***In vitro* and *in vivo* studies-plasticity of BMDCs**

Multiple and elegant studies from independent groups have shown quite clearly that bone marrow stem cells can differentiate along multiple diverse lineage pathways^[14-17]. These findings challenge the conventional view that bone marrow stem cells give rise only to the marrow mesenchyma or formed elements in the peripheral blood. *In vitro*, BMDCs have been shown to differentiate at the single cell level and acquire characteristics of mesoderm, neuroectoderm and endoderm^[15,16]. These cells appear to use culture environmental factors to guide lineage decisions. Strikingly, *in vivo* studies in the mouse model have confirmed this plasticity. Elegant studies utilizing transplanted single cells demonstrate differentiation along multiple lineages, supporting a central role for the local tissue environment in dictating differentiation of stem cells, confirming that a single cell is multipotent, and supporting the assertion

that experimental findings demonstrating multiple cell lineage differentiation is not due to circulating tissue specific progenitor cells, but rather to a single multipotent cell. In these studies, multiple types of epithelial cells have been shown to be derived from BMDCs including epithelium of the lung, gastrointestinal tract and skin after transplantation of a single bone marrow-derived stem cell^[14]. This is not a transient event, as cells can be recovered nearly a year after transplantation. In the gastrointestinal tract, engrafted cells are seen as isolated epithelial cells in the gastric pits of the stomach, the small intestinal villi, the colonic crypt, and rarely in the esophagus. Under these experimental conditions, cells were recovered as single differentiated epithelial cells, and did not appear to engraft into the stem cell niche as clonal expansion was not seen. Infusion of labeled BMDCs into a non-irradiated host, also led to the engraftment (*albeit* to a lesser degree) and differentiation as epithelial cells of the liver, lung and gut in a similar pattern to that seen with marrow ablation and transplantation^[15], demonstrating that engraftment and differentiation are true physiological events and not merely artifacts of irradiation and experimental manipulation. While epithelial cell damage is not necessary for engraftment, studies support the notion that damage to the epithelium increases engraftment.

The mechanism by which the marrow-derived cells acquire the appropriate phenotype of epithelial cells is not known, with evidence supporting both direct differentiation or fusion with a peripheral cell^[18-21]. The method of engraftment and differentiation may be specific to the individual tissues and/or may depend on the mechanism of injury inducing engraftment. Irrespective of the mechanism involved, BMDCs have been shown to engraft and take on the function of cells within the peripheral tissues^[16,18-23].

HUMAN STUDIES - EVIDENCE FOR PLASTICITY OF BMDCS IN PATIENTS TRANSPLANTED WITH GENDER MISMATCHED BONE MARROW

Human studies have confirmed that plasticity of BMDCs is not restricted to mice, and may be a physiologically relevant phenomenon in man as well. Studies, examining peripheral tissue of female patients transplanted with bone marrow derived from male donors, have shown that BMDCs from the donor can differentiate into skin, gut epithelium and mature hepatocytes^[24,25]. Identification of the Y chromosome in cells of these tissues confirms that BMDCs can substantially repopulate the GI tract epithelium^[25], and this repopulation does not appear to be a rare event. Patients in these studies have some level of graft-versus-host disease, and the level of inflammation in the tissue correlates with the level of donor cell engraftment. These findings are consistent with the data derived from murine studies and suggest the inflammatory environment is crucial for optimal engraftment and differentiation of BMDCs. The fact that BMDCs have the capacity to differentiate along organ-specific lineages appropriate for the organ of engraftment, and are found in increasing num-

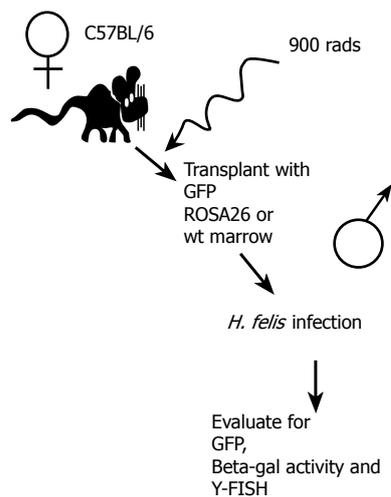


Figure 3 An experimental mouse model for bone marrow transplantation and *H. felis*-induced gastric carcinoma.

bers during chronic inflammation (a condition associated with cancer), places these cells “in the right place, at the right time” to be candidates for the cancer stem cell.

Similarities between BMDCs and cancer cells

In addition to what appears to be of immense plasticity of cells within the bone marrow, BMDCs have other traits which make them attractive candidates for cancer stem cells. BMDCs have the capacity for self renewal, are long lived, are chemoresistant, and may be inherently mutagenic^[26-29]. Intriguing is the fact that similar growth regulators and control mechanisms are involved in both cancer and stem cell maintenance. For example, proteins from the polycomb group, the epigenetic chromatin modifiers, are involved in both cancer development and maintenance of embryonic and adult stem cells^[30]. Also, pathways used by bone marrow stem cells for trafficking appear to be exploited by tumor cells for metastasis^[31]. For instance, chemokines and cytokines produced during chronic inflammation (such as SDF-1) influence the behavior and migration of cancer cells. These are the same chemokines and cytokines responsible for physiological stem cells homing back to the marrow cavity^[32-36]. Identification of bone marrow stromal cell-derived growth inhibitor as a potent inhibitor of breast cancer cell migration, and the capability of this protein to induce cell cycle arrest and apoptosis in breast cancer stem cells further supports the use of similar growth mechanisms between stem and cancer cells^[37]. Inflammation of the GI tract is associated with IL-6 and IL-8 production which initiate neutrophil infiltration^[39]. Interestingly, IL-6 is also chemotactic for MSC^[33]. Other cytokines and chemokines prominent in the setting of mucosal inflammation such as VEGF and MIP-1 α are also chemoattractants for MSC^[33,34]. Receptors such as CXCR 2 and 4 are found on both cancer cells and stem cells, and influence the homing of stem cells, or invasion/metastases of cancer cells, suggesting a link between the two populations of cells. One might suppose that a mechanism similar to that used to regulate BMDC circulation and homing back to areas of bone may also facilitate migration and engraftment of BMDCs into peripheral tissues as a result

of chronic inflammation, if the peripheral tissue secretes the appropriate homing signals.

Additionally, immune escape has long been a perplexing property of cancer cells; MSCs have unique immunological properties in that they are not immunogenic, they do not stimulate alloreactivity, and they escape lysis by cytotoxic T cells and natural killer cells^[39]. This inherent ability to evade immune recognition may explain why many cancer cells evade the host immune response.

BMDCs as the origin of epithelial cancer: helicobacter induced gastric cancer as a model system

We reasoned that BMDCs, as the ultimate uncommitted adult stem cell, might represent the ideal candidate for transformation, if placed in a favorable environment. We used the well-described *H. felis*/C57BL/6 mouse model of gastric cancer to test this theory^[40]. This model is optimal for studying the role of stem cells in inflammatory-mediated cancers because C57BL/6 mice do not develop gastric cancer under controlled conditions. With *Helicobacter* infection, however, the gastric mucosa progresses through a series of changes including metaplasia and dysplasia, culminating in gastrointestinal intraepithelial neoplasia (GIN)^[41] by 12-15 mo of infection, thus reiterating human disease, where gastric cancer in the absence of *Helicobacter* infection is unusual, while longstanding infection carries a significant (up to 1-3%) risk of gastric cancer^[42-47]. In order to test the role for BMDCs in gastric cancer (Figure 3), C57BL/6J mice were myeloablated and transplanted with gender-mismatched bone marrow from mice that expressed a non-mammalian beta-galactosidase enzyme [C57BL/6J *Gtrosa26* (ROSA 26)], mice that expressed green fluorescent protein [C57BL/6J *-beta-actin-EGFP* (GFP)], or control C57BL/6J litter mates. Engraftment of ROSA26 BMDCs into the gastric mucosa was confirmed by several independent methods including detecting enzyme activity, specific B-galactosidase immunohistochemistry (IHC, two cytoplasmic markers) (Figures 4 and 5), and detection of LacZNeo fusion gene sequence (nuclear marker) by PCR within beta-galactosidase positive gastric glands isolated by laser capture microscopy. In those mice transplanted with GFP marrow, GFP was detected by fluorescence activated cell sorting of cytokeratin positive-single cell preparations, and GFP immunohistochemistry of tissue sections. X and Y chromosome fluorescent *in situ* hybridization (X and Y-FISH) was used as an additional means to detect BMDCs in gender mismatched transplants^[40].

As expected, acute *Helicobacter* infection was associated with an influx of bone marrow-derived inflammatory cells (Figure 4A - blue staining) into the tissue. At early time points, we did not detect any engraftment or differentiation of BMDCs to an epithelial cell phenotype. At 20 wk of infection, rare glands entirely replaced by BMDCs were isolated, suggesting that engraftment into the stem cell niche had occurred. These findings were more pronounced at 30 wk, where antralized glands and metaplastic cells at the squamocolumnar junction were entirely replaced by marrow-derived cells (Figures 4D and 4E). The severity of intraepithelial dysplasia increased over time, and by one year of infection, most mice developed invasive neoplastic glands. All of the intraepithelial neoplasia in mice infected

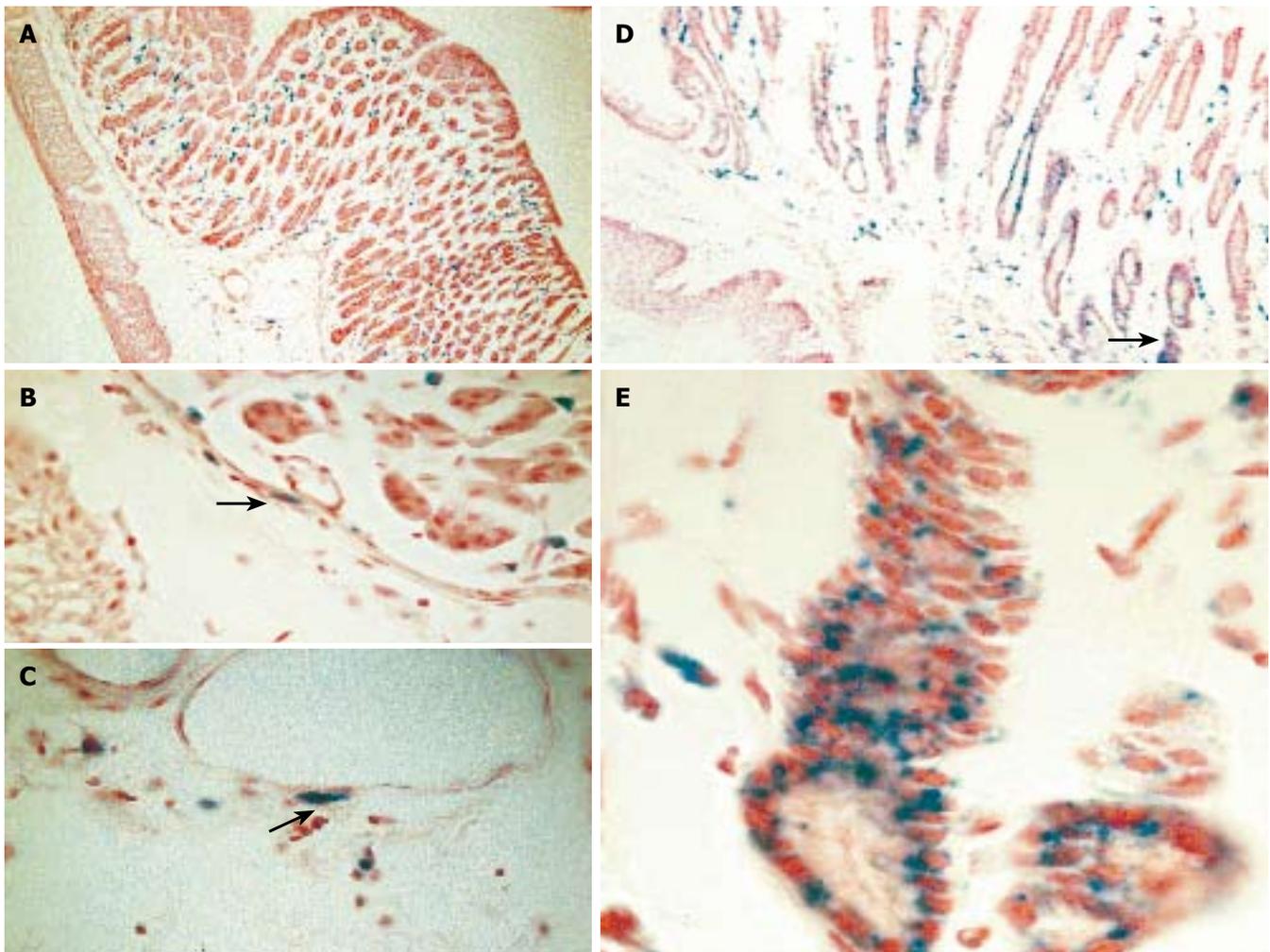


Figure 4 Engraftment of donor-derived ROSA-26 marrow by x-gal staining. **A:** Mice transplanted with ROSA 26 marrow and infected with *H. felis* for 4 wk had donor-derived leukocytes (blue) infiltrating the gastric mucosa, and no engraftment into gland structures. **B and C:** A higher power view reveals myocytes and myofibroblasts in the sub-mucosal tissue adjacent to vascular structures (arrows). **D:** After 30 wk of infection, marked architectural distortion is seen with antralization and appearance of metaplastic glands. Entire gland structures are derived from donor marrow (blue staining). Gland shown in panel D (arrow) is shown at higher power in **E**.

for 12-16 mo rose from donor marrow cells, strongly suggesting an inherent vulnerability of this population of cells to malignant progression. Progressive parietal and chief cell loss is a hallmark of chronic *Helicobacter* infection. Of the few parietal or chief cells which we isolated from the infected mice, none were derived from the bone marrow, strongly suggesting that marrow cells do not differentiate toward the parietal or chief cell phenotype under the experimental conditions that were used^[40].

Normal healing of the gastric mucosa after iatrogenic ulceration likewise did not require BMDCs^[40], nor did loss of specific cell lineages, such as targeted ablation of parietal cells, lead to marrow engraftment^[40]. Rather, it seems that long standing inflammation and inflammatory mediated damage to the epithelium is required - an environment strongly linked to the development of cancer in many settings. In our *Helicobacter*-gastric cancer model, infection and inflammation reached a plateau at 8 wk; however, engraftment was not apparent until 20 wk, suggesting that events other than increased inflammation are responsible for engraftment. Between 8 and 20 wk, there is loss of the oxyntic glands, and a restructuring of the gastric architecture to include metaplastic cell lineages, re-

flecting the effects of an abnormal tissue milieu on rapidly proliferating cells^[48]. Once engraftment began, however, the number of bone marrow-derived glands increased dramatically, suggesting that a threshold for recruitment had been reached^[40].

In addition to epithelial cells within the tumor, BMDCs also comprise a subset of cells within the tumor stroma and within seemingly uninvolved epithelium and subepithelial spaces adjacent to the tumors. We have recovered adipocytes (Figure 5C), fibroblast, endothelial cells and myofibroblasts (Figures 4B and 4C) derived from bone marrow precursors in areas adjacent to dysplasia and neoplasia.

Based on these experiments, we have proposed a new paradigm for epithelial cancer (Figure 6). Chronic tissue inflammation leads to tissue injury and with time, to tissue stem cell failure. Peripheral stem cell failure leads to recruitment and permanent engraftment of BMDCs into the tissue stem cell niche, where the BMDCs essentially take over the function of the tissue stem cell. In the setting of inflammation, specifically with Th1 type cytokines and an abnormal tissue environment (for example, one lacking chief and parietal cells), the BMDCs initiate differentia-

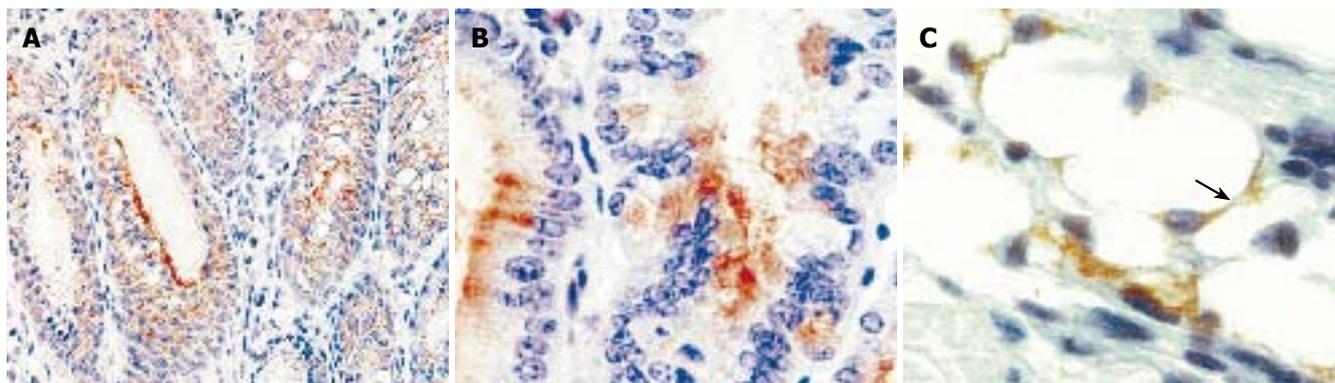


Figure 5 Immunohistochemistry for bacterial beta-galactosidase confirms uniform signal in gastrointestinal neoplasia. Mice developed severe dysplasia and intraepithelial neoplasia derived from donor marrow, 12-15 mo after infection with *H. felis* (A) and (B). Immunohistochemistry for bacterial beta-galactosidase demonstrates cytoplasmic staining in dysplastic glands. A population of adipocytes in the submucosa are also stained for beta-galactosidase (arrow).

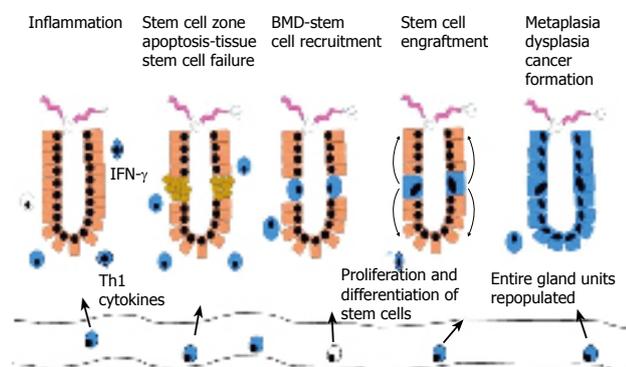


Figure 6 A new paradigm proposed for epithelial cancer.

tion, but fail to regulate growth programs appropriately and progresses through stages of metaplasia and dysplasia. We speculate that the inappropriate retention of primitive growth programs in a stem cell forced to replicate may permit survival despite otherwise lethal mutations, thus allowing transformation. This new model brings together previously unexplained observations regarding the behavior of cancer, and presupposes that properties inherent to cancer such as their resistance to apoptosis, their unlimited growth potential and their ability for local spread and distant metastasis are fundamental to the origin of the cell, rather than traits acquired. The concept of cancer initiation and promotion can also be viewed within the context of this model. Initiation may represent BMDCs trafficking into the stem cell niche as a result of tissue stem cell damage. In the absence of continued inflammation and injury, these engrafted cells may behave in a way indistinguishable from endogenous tissue stem cells. Promotion may represent an additional stimulus received at a later time that allows sustained proliferation of BMDCs and transformation.

***In vitro* experiments and animal models supporting the BMDC-epithelial cancer model**

In addition to the *Helicobacter*-gastric cancer model, other studies have begun to address the role of BMDCs in cancer using various *in vitro* and *in vivo* models. For example, BMDCs have been shown to localize to a known stem cell

niche within the epidermis known as the CD34 positive bulge region of the hair follicle, and clonally expand to repopulate portions of the epidermis, functioning as an epidermal stem cell^[49]. Similar to our findings, engraftment of BMDCs to the stem cell niche is dramatically increased with injury severe enough to deplete peripheral stem cells in the region. However, these are short-term studies. Longer term studies utilizing carcinogen exposure will determine the eventual fate of these BMDCs, and determine if BMDCs in the stem cell niche behave differently from peripheral stem cells occupying the same niche. It is intriguing, however, to speculate the ultimate fate of these stem cells given the prevalence of BMDC-skin carcinoma in solid organ recipients (see below).

In addition to residing in the epithelial stem cell niche, bone marrow-derived myofibroblasts have been recovered within the colonic subepithelial compartments in both mice and human beings^[50,51]. Interestingly, Direkze *et al.* observed that in the IL-10 knockout mouse model of colitis, up to 45% of subepithelial myofibroblasts were marrow derived^[51], suggesting that in the setting of chronic inflammation, damaged tissue is replaced by BMDCs. When the same group looked at tumor-associated myofibroblasts and fibroblasts, they also found a significant portion of these cells derived from bone marrow cells^[50]. It is not clear from these data, if tumors recruit bone marrow cells into the stromal compartment or if resident myofibroblasts and fibroblasts derived from marrow contribute to tumor formation because of abnormal signaling behavior.

Adenocarcinoma of the distal esophagus (Barretts' adenocarcinoma) results from reflux-induced mucosal damage followed by healing with a metaplastic intestinal cell lineage. This intestinal metaplasia is prone to malignant degeneration and is another ideal model to test the role of BMDCs in inflammatory-mediated cancers. Using a rat model of Barrett's metaplasia, a significant contribution of BMDCs to the stroma and the metaplastic epithelium has been demonstrated, supporting a role for BMDCs in these pre-neoplastic lesions^[52]. Though these findings have only been reported in an abstract form so far, this information is especially exciting because it provides evidence of direct BMDC involvement in carcinogenesis from both an additional species (rat) and tissue type (esophagus), providing

further support for our BMDC-epithelial cancer model.

Human data supporting the BMDC-epithelial cancer model

In human beings, the incidence of solid tumors is significantly increased following bone marrow transplantation^[53] and may be related to persistent chronic inflammation of graft *vs.* host disease. The data on BMDCs in human cancers, however, have been conflicting. First, it is difficult to examine the contribution of donor marrow to tumor formation in human beings because of a paucity of cell markers to consistently identify autologous BMDCs or donor cells after BM transplantation. The most reliable marker we have to date is identification of the sex chromosomes in sex mismatched transplants. However, there are inherent difficulties with using Y-chromosome identification. X/Y fluorescent *in situ* hybridization (FISH) analysis of archived tumors is estimated to miss more than 50% of Y-positive cells due to sectioning bias, where only a portion of the nucleus and thus only a portion of the chromosomes are included in the tissue section. Additionally, females with a history of carrying a male fetus may show peripheral blood chimerism confounding interpretation of data, and eliminating this population from the study. Also, tumors identified within a short time after transplant may reflect the effects of immunosuppression on previously undetected early malignancy and not newly formed tumors, and may explain why some studies conclude tumors in these patients are host derived^[54], while other studies demonstrate a definite contribution of donor's-BMDCs^[55]. Studies utilizing larger numbers of patients followed for longer periods of time will better address this new and controversial area, and determine if the BMDCs are confined to the stroma, involved in angiogenesis or constitute the epithelial component of the tumor mass in human beings.

In addition to patients receiving bone marrow transplants, recipients of solid organ transplants also have a higher incidence of secondary malignancy. Interestingly, in solid organ transplant recipients, hematopoietic cells of donor origin are often found in the circulation, indicating that hematopoietic stem cells are transferred with the transplanted organ^[56,57]. These transferred stem cells have been shown to give rise to Kaposi sarcoma (KS), a vascular tumor^[58], and skin carcinoma^[59]. The detected KS lesions occurred distal to the graft site, and formed presumably via mobilization of donor progenitor cells with subsequent transformation at a distant site. Donor-derived stem cells contribute to skin carcinomas, and have been recovered as components of squamous cell carcinoma, basal cell carcinoma, actinic keratosis, keratoacanthomas and benign cutaneous lesions^[59], attesting to the great potential for abnormal differentiation of these cells. BMDCs as terminally differentiated cells in other organs including hepatic endothelial cells, hepatocytes and biliary epithelial cells^[60], suggesting that these cells may play a role in transformation within these organs as well, if subjected to the appropriate environmental conditions.

CONCLUSION

One of the greatest and most elusive challenges in cancer biology has been to identify the cellular origin of cancer.

We have identified the bone marrow stem cell as the cell of origin of Helicobacter-induced gastric cancer in a mouse model, radically altering our current view of gastric cancer formation in particular, and of inflammation-mediated cancers in general. The concept of BMDC plasticity is being increasingly recognized and validated by independent groups. Our recent observation that BMDCs are the origin of Helicobacter-induced gastric cancer^[40] combined with supporting observations of BMDCs in other tumors such as benign and malignant tumors of the skin^[59], Kaposi sarcoma^[58] Barretts' adenocarcinoma of the esophagus^[52] as well as demonstration of BMDCs as constituents of tumor stroma and tumor vascular structures^[51-55] suggests exciting approaches for cancer therapy. If the propensity for BMDCs to transform is based on inappropriate regulation of immature growth programs, with growth programs left "turned on" rather than the previously held concept of mutation driven-reactivation of programs, can we target these pathways? Undoubtedly, genetic mutations have occurred which are irreversible; but if we can target and switch off inappropriately activated growth cascades, perhaps we can push these damaged cells into apoptosis or enhance the sensitivity to conventional chemo- and radiotherapy. These approaches may lead to novel and more efficacious cancer therapy.

Presently, our laboratory is involved in identifying the cell population within the bone marrow capable of cancer formation as well as defining the homing and differentiation signals which allow these cells to access to gastric mucosa, and to differentiate as metaplastic and dysplastic cells. Studies designed to determine if fusion is a means of bone marrow cell integration into gastric mucosa and gastric cancer are underway. The applicability of these findings to other epithelial cancers will be tested as well as our ability to control the growth of these cells by manipulations of the local tissue environment. These efforts are aimed at identifying cell-specific targets for chemotherapy. Findings from these studies will radically alter our approach to the treatment of gastric cancer as well as other solid tumors, and offer hope for improved survival and potential cure.

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REVIEW

Current role of surgical therapy in gastric cancer

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Abstract

Surgery is currently the only potentially curative treatment for gastric cancer. Since the inception of the gastrectomy for cancer of the stomach, there has been debate over the bounds of surgical therapy, balancing potential long-term survival with perioperative morbidity and mortality. This review delineates the current role of surgery in preoperative staging, curative resection, and palliative treatment for gastric cancer.

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Key words: Gastric; Cancer; Surgery

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INTRODUCTION

Gastric cancer is one of the most common malignancies in the world and is a leading cause of cancer death. Despite some recent advances in neoadjuvant therapy, studies generally have failed to show any improvement in overall or relapse-free survival following adjuvant therapy. Surgical therapy remains as the most effective modality in treating gastric cancer. The goal of this review is to evaluate the optimal role and impact of surgery in regards to staging, resection of primary, regional and locally advanced disease, and palliation of symptoms in patients with incurable gastric cancer.

STAGING

Laparoscopy has emerged as an essential staging modality prior to gastric resection, identifying unresectable disease in a significant number of patients deemed resectable by current radiographic and endoscopic modalities. The

diagnostic yield of laparoscopy has been improved by the addition of laparoscopic ultrasound and peritoneal cytology.

Stell *et al*^[1] compared 103 patients with gastric adenocarcinoma who underwent preoperative staging with ultrasound (US), computed tomography (CT), and laparoscopy. Histologic confirmation was obtained at laparotomy ($n=65$) or with percutaneous liver biopsy during laparoscopy ($n=27$). The sensitivity, specificity, and accuracy of laparoscopy in detecting hepatic metastases ($n=27$) was 96%, 100%, and 99%, respectively. Laparoscopy had a sensitivity of 53%, a specificity of 100%, and an accuracy of 65% in detecting nodal metastasis ($n=49$). Peritoneal metastases were histologically confirmed in 13 patients, and laparoscopy was 69% sensitive, 100% specific, and 94% accurate.

Burke *et al*^[2] reviewed 103 patients who were deemed free from intra-abdominal metastases by CT scan and subsequently underwent laparoscopy. A control group who underwent laparotomy for possible gastric resection and subsequently found to have M1 disease was also reviewed. Laparoscopy accurately staged 94% of these patients. Histologic evidence of metastasis was obtained in 32 patients during laparoscopy. Of the 71 patients who were M0 by laparoscopy, 65 were confirmed by laparotomy. Of the six false negatives, three were missed peritoneal metastases, and three were missed distant nodal metastases, two of which were identified after resection. Of the 32 patients who were M1 on laparoscopy, 4 had chemotherapy followed by resection, 3 had further palliative procedures, and 1 had a mini-laparotomy for additional specimen. The remaining 24 patients went on to have no further operation. When comparing these 24 patients to the control group of 60 patients with M1 disease on laparotomy, the hospital stay was significantly shorter. There was one complication in the laparoscopy group and eight in the laparotomy group; however, this did not reach significance. The authors concluded that laparoscopy is a valuable staging tool that can spare patients with asymptomatic M1 disease a laparotomy, and identify those patients who may benefit from neoadjuvant therapy.

The addition of laparoscopic US has improved the yield of laparoscopy for staging gastric cancer. Hulscher *et al*^[3] reviewed the utility of laparoscopic US in staging 48 patients with gastric carcinoma of the cardia and esophageal invasion. All of these patients were staged with endoscopic US, percutaneous US of the neck and abdomen, conventional chest radiograph and bronchoscopy if there was extensive proximal invasion. Metastatic

disease was detected and histologically confirmed in 11 patients (23%), 7 detected with laparoscopy alone, and an additional 4 detected by laparoscopic US. Of the patients who went on to laparotomy, two were found to have posterior liver metastases, and two to have unresectable lymph nodes at the base of the celiac axis. Three of these four lesions were detected laparoscopically, but were either not amenable to biopsy due to position, or were negative on laparoscopic biopsy. The authors concluded that laparoscopy is a valuable staging tool for gastric adenocarcinoma of the cardia, and that the addition of US increases the yield, avoiding laparotomy in 23% of patients.

Peritoneal cytology obtained at laparoscopy has proven to be a predictor of post-operative mortality in patients undergoing curative (R0) resection. Bentrem *et al*⁴¹ reviewed 371 patients who underwent staging laparoscopy with peritoneal washings and subsequent R0 resection as a combined procedure. Twenty-four patients (6%) had positive cytology, defined as the presence of adenocarcinoma cells, regardless of quantity. Positive cytology without evidence of macroscopic M1 disease was associated with advanced preoperative T stage and advanced AJCC stage, but not preoperative N stage. The median survival for patients with positive cytology was 15 mo, compared to 98.5 mo for those with negative cytology. On multivariate analysis, preoperative T stage, preoperative N stage, primary tumor site, and peritoneal cytology were significant predictors of survival, with positive cytology showing the highest risk ratio. The authors have altered their practice in response to this information, performing staging laparoscopy as a separate procedure in patients considered high risk (T3/4 and AJCC stage II/III). Those patients found to have positive cytology were then referred for chemotherapy followed by possible resection with intraperitoneal chemotherapy.

Laparoscopy and laparoscopy US are currently accepted as standard pre-operative staging tools. Laparoscopy is significantly more accurate than US and CT in detecting hepatic, nodal and peritoneal metastases. Reports suggest minimal added morbidity and no mortality from laparoscopy. Peritoneal cytology is not widely accepted as a screening tool; however, this is a topic of growing research interest.

EXTENT OF GASTRIC RESECTION

Distal gastric adenocarcinoma

The extent of gastric resection for distal lesions had been debated, and the traditional view that total gastrectomy (TG) is required for all gastric lesions has been challenged. Gouzi *et al*⁵¹ conducted a multicenter randomized trial comparing TG to subtotal gastrectomy (SG) enrolling 169 patients with resectable lesions of the gastric antrum. Patients with macroscopic lymph node involvement of the cardioesophageal or splenopancreatic region were excluded from the study. TG consisted of TG with Roux-en-Y esophagojejunostomy. Splenectomy was not routinely performed; however, the TG group had an unspecified higher splenectomy rate than the SG group. SG consisted of a distal gastrectomy and Billroth II gastrojejunostomy.

Both procedures included a total omentectomy, and lymph node dissection extended to the pyloric, left gastric, hepatic, and cardiac nodes. The groups were well matched for tumor size, extent of invasion, and lymph node stage. There was a post-operative mortality of 2.4%, with three deaths in the SG group and one death in the TG group. The non-lethal complication rate was 33% in the TG group and 34% in the SG group. Lymph node involvement and serosal extension were significantly associated with 5-year survival; however, extent of resection was not. The authors concluded that TG or SG could be performed with equal morbidity and mortality, but that TG offered no added survival benefit. A major criticism of this study was that it was underpowered to detect a difference between the groups, as 200 patients were originally calculated to show a difference of 20%.

In a larger trial, Bozzetti *et al*⁶¹ also performed a randomized trial comparing TG to SG in 618 patients with resectable tumors at least 6 cm from the cardia. Patients were enrolled if they had tumors of the distal half of the stomach without evidence of metastatic disease, were under 75 years of age and in good health, and had no previous gastric resection or chemotherapy. Patients were further assessed at laparotomy for a minimum distance from the tumor to the cardia and no evidence of peritoneal, D3 lymph node metastasis or extension into adjacent organs. Patients were randomized intra-operatively to TG or SG. Both operations were performed with a D2 lymphadenectomy. Splenectomy was left to the discretion of the operating surgeon. Patients were then followed regularly for a median follow-up of 72 mo in the SG group and 75 mo in the TG group. Five-year survival was 65.3% in the SG, and 62.5% in the TG group, suggesting that type of resection had no influence on the survival. Site of tumor, wall invasion, extension of surgery including splenectomy, and relative frequency of metastatic lymph nodes were significant predictors of survival. Given the lack of survival benefit, the authors concluded that SG was the preferred operation for distal gastric cancer, provided that a proximal margin of at least 6 cm could be obtained, because it is technically less demanding, results in a lower splenectomy rate, and is associated with better quality of life (QOL).

QOL is a very important outcome after gastrectomy. For tumors of the distal stomach, given the current data suggesting equivalent morbidity and survival for TG and SG, the procedure with the best QOL profile is preferred. Davies *et al*⁷¹ compared the QOL of patients following TG to those after SG. These authors compared 46 patients who had undergone an R0 resection. Twenty-six underwent TG and 21 underwent SG. Five questionnaires were used: the Rotterdam symptoms checklist, the Trold index, the hospital anxiety and depression scale, the activities of daily living score, and the Visick grade. Patients were questioned before the operation and at 1, 3, 6, and 12 mo. There was no difference in QOL prior to the operation or at 1, 3 or 6 mo; however, patients undergoing SG had a significantly higher QOL at 1 year. The authors thus concluded that, given equivalent survival, SG is the operation of choice for tumors of the distal stomach.

Based on data that accrued in prospective, randomized

trials, it is now accepted that SG is an appropriate oncologic operation for distal adenocarcinoma.

Proximal gastric adenocarcinoma

The extent of resection needed to achieve cure in tumors of the gastroesophageal junctions has been a topic of much debate. Ito *et al*^[8] reviewed patients with Siewert type II or III carcinoma of the gastric cardia in an attempt to discern the optimal surgical approach. Eighty-two patients were included in the study, 59 with type II and 23 with type III lesions. The surgical approach varied, with 33% undergoing total esophagectomy, 29% undergoing extended gastrectomy with thoracotomy, and 38% undergoing extended gastrectomy without thoracotomy. There was no significant difference in the type of procedure between type II and III lesions. There was no significant difference in post-operative mortality; however, there was a higher post-operative morbidity associated with total esophagectomy as compared to extended gastrectomy with or without thoracotomy (33% *vs* 11%). The addition of a thoracotomy to extended gastrectomy did not have an impact on post-operative morbidity (13% *vs* 10%). There was a significantly higher incidence of microscopic residual disease at the proximal margin in the extended gastrectomy group with or without thoracotomy as compared to the total esophagectomy group (38% *vs* 7%). The mean follow-up was 34 mo and 5-year survival for the entire group was 33%. Multivariate analysis revealed that patient's age over 65, lymph node metastases, and absence of an R0 resection were adversely associated with survival. There was no survival difference between the different types of resection despite a higher incidence of positive proximal margins in the extended gastrectomy group. Based on these results, showing R0 status and nodal status to be predictors of survival, the authors made the following recommendations: (1) A minimum proximal margin of 6 cm and distal margin of 4 cm should be obtained. (2) A minimum of 15 lymph nodes should be sampled. The type of surgical approach should be tailored to fit the individual patient with these goals in mind.

TG is the traditional treatment for proximal gastric cancer; however, this has been recently challenged as well. Harrison *et al*^[9] reviewed 98 patients with proximal gastric cancer who underwent gastric resection via an abdominal approach, excluding all patients who underwent esophagogastrectomy. Of these 98 patients, 65 underwent proximal gastrectomy (PG), and 33 underwent TG. There was no difference in post-operative mortality (6% *vs* 3%). Post-operative morbidity data was not analyzed; however, there was no difference in the length of hospital stay. There was no significant difference in time to recurrence or first site of recurrence. The 5-year survival rate was not significantly different between the TG and PG groups with a mean follow-up of 30 mo (43% for PG *vs* 41% for TG). The authors concluded that either procedure can be performed safely without sacrificing long-term survival.

The optimal approach for carcinoma of the gastroesophageal junction or gastric cardia is still a topic of debate. The over-riding factor, regardless of the type of resection, is the ability to obtain a margin-negative resection.

EXTENT OF LYMPH NODE DISSECTION

The extent of lymphadenectomy is one of the most controversial topics in gastric cancer surgery. The Japanese developed an extensive classification system for the regional lymph nodes and a systematic method of dissection referred to now as the D2 resection. This method is described in detail by Maruyama *et al*^[10], who also reviewed the Japanese National Cancer Center experience with gastric resection over the period from 1963 to 1985. The authors reported an improvement over this time span in 5-year survival for resected patients from 44.3% to 61.6%. Most of this improvement was attributed to early gastric cancer screening established at a national level; however, they do report an increase in survival over this time period with respects to specific stage, T stage, and N stage.

Shiu *et al*^[11] reviewed the results of gastric resection and lymphadenectomy for gastric cancer at Memorial Sloan Kettering Cancer Center over a 20-year period. They analyzed the outcomes of 210 patients and attempted to identify risk factors for mortality. On multivariate analysis, they identified non-pyloric tumor site and greater than three positive lymph nodes as pathologic risk factors. They also identified positive microscopic margins, inadequate lymphadenectomy, and TG as independent surgical risk factors for mortality. Adequate lymphadenectomy was defined as one and a half lymph node levels beyond the furthest level of lymph node disease. For example, patients with N0 disease would require a dissection including the N1 and part of N2 nodes.

This data from a Western center as well as the Japanese data were cited to justify extended lymphadenectomy, a practice which was not commonplace in the West. Multiple clinical trials were performed to look at the morbidity, mortality, and long term survival of a D2 dissection in Western centers.

The best known trial to evaluate lymphadenectomy was from the Dutch Gastric Cancer Group. Bonenkamp *et al*^[12] published their results from a randomized controlled trial comparing D1 and D2 gastrectomy in 80 Dutch hospitals over 5 years. D1 resection was defined as containing only the N1 (perigastric) nodes. D2 resection was defined as encompassing the N2 nodes. The spleen and distal pancreas were resected in 11 (3%) of D1 resections and 30 (37%) of D2 resections. The type of gastrectomy, TG *vs* SG, as well as the method of reconstruction were left to the preference of the surgeon. Surgeons were instructed in the technique with video classes, and eight visiting Japanese surgeons were present to supervise all D2 resections. Seven hundred and seven patients underwent resection with intention to treat, and 632 were found to have an R0 resection, and thus followed for evidence of recurrence. These authors reported a statistically significant difference in post-operative mortality (4% *vs* 10%) and complication rate (25% *vs* 43%) for D1 *vs* D2 resection. The 5-year survival rate was 45% for the D1 group and 47% for the D2 group, which was not statistically significant. The 5-year risk of relapse was 43% in the D1 group and 37% in the D2 group, which also was not statistically significant. Subgroup analysis, however, showed that in patients who

did not require splenectomy or pancreatectomy, there was a significantly lower 5-year risk of relapse. The conclusions drawn from this article were that D2 lymphadenectomy was associated with a higher morbidity and mortality without offering a long-term survival benefit, and thus it was not recommended. The sub-group analysis also questioned the benefit of distal pancreatectomy or splenectomy.

A concomitant British trial was also performed over the same time period. Cuschieri *et al*^[13] reported the results of a randomized study comparing D1 to D2 resections. Four hundred patients were randomized. In this trial a D1 resection consisted of the removal of all lymph nodes within 3 cm of the tumor and D2 resection consisted of the standard resection of the omental bursa, the hepatoduodenal nodes for antral lesions and the splenic artery, splenic hilar, and retropancreatic nodes by distal pancreatectomy for middle and upper third lesions. They reported similar post operative morbidity and mortality data to that of the Dutch study for the D2 resection group, and showed no difference in the 5-year survival (35% *vs* 33%) or risk of recurrence. As in the Dutch trial, the British trial showed that resection of the spleen and pancreas was independently associated with decreased survival.

Both of these large randomized studies showed no benefit from a D2 resection; however, each also raises the question: is there a role of D2 resection without pancreatico-splenectomy? Two current studies attempt to answer that question.

Edwards *et al*^[14] compared D1 resection to D2 resection sparing the pancreas and spleen in a total of 118 patients. The study was not randomized, as one surgeon performed all the D1 resections ($n=36$) at one hospital and the other surgeon performed all the D2 resections ($n=82$) at another hospital in the same region of Wales. Splenectomy and pancreatectomy were performed only for evidence of direct invasion of tumor. They reported similar operative mortality (8.3% *vs* 7.3%) and a significant 5-year survival advantage for the D2 resection group (32% *vs* 59%), which was most evident in the patients with stage III disease (8% *vs* 33%). Extent of lymphadenectomy was independently associated with increased survival on multivariate analysis. The authors concluded that a modified D2 resection sparing resection of the pancreas and spleen offers a survival advantage over a D1 resection with no increase in short-term morbidity or mortality.

Recent phase II data from a multicenter trial conducted in Italy showed acceptable morbidity and mortality with good 5-year survival following pancreas-preserving D2 resection^[15]. The Italian Gastric Cancer Study Group enrolled 191 patients, and pancreas-preserving D2 resection was performed with an operative mortality of 3.1%, morbidity of 20.9%, and 5-year survival rate of 55%. These numbers compare favorably to the Japanese experience, and suggest that pancreas-preserving D2 may offer a survival benefit; however, the randomized comparison to D1 resection has not yet been performed.

Data from Japan concerning mortality and 5-year survival after gastrectomy had been criticized for being retrospective in nature. A D1 gastrectomy is considered

as a non-curative operation in Japan; thus, it would not be performed in any randomized trial. Sano *et al*^[16] are conducting a randomized trial comparing standard D2 resection to extended para-aortic lymphadenectomy. This is the first randomized prospective study to come from Japan. The selection criteria of the surgeons and the selected centers should be mentioned. Only surgeons with over 100 D2 gastrectomies, and only centers with over 80 gastrectomies a year were selected for the trial. This is in stark contrast to the Dutch and British trials which did not require a set training period to master the “learning curve” of the procedure. The spleen was resected in all the cases where TG was performed, and the pancreas was resected only in cases of direct invasion. The morbidity and mortality data for this trial have been released. The overall morbidity of a D2 resection was 20.9%, with a mortality of 0.8%. The conclusions that can be drawn from this study, pending the 5-year survival data, are that D2 resection can be performed at specialized centers by surgeons with ample experience with low morbidity and mortality.

Based on the current data, it appears that a modified D2 lymphadenectomy, sparing the spleen and pancreas when possible, can be performed safely and may offer the best chance for long-term survival; however, the randomized controlled data to support this argument is currently in progress.

EXTENDING RESECTION TO ADJACENT ORGANS

D2 resection with splenectomy and distal pancreatectomy was performed by many centers in Japan to facilitate dissection of lymph nodes around the splenic artery with early reports suggesting improved survival^[10]. The utility of extended resection was later called into question by many Japanese and Western surgeons. Otsuji *et al*^[17] reviewed 128 patients who underwent TG for gastric adenocarcinoma of the middle or proximal stomach. Of these, 35.9% underwent pancreaticosplenectomy, 44.6% splenectomy, and 19.5% gastrectomy alone. Pancreaticosplenectomy increased the risk of pancreatic fistula significantly. Five-year survival for the pancreaticosplenectomy group, the splenectomy group and the gastrectomy alone group were 40.7%, 55.9%, and 54.2%, respectively; however, on multivariate analysis pancreaticosplenectomy and splenectomy alone were not independently associated with survival. The conclusions drawn from this study were that extension of TG to include pancreaticosplenectomy or splenectomy increases the risk of complications without improving survival.

Kasakura *et al*^[18] reviewed 1 938 gastric resections over 18 years and also concluded that splenectomy and distal pancreatectomy do not have an impact on survival and are associated with an increased incidence of complications. Of these 1 938 patients, 78 underwent splenectomy (S), 105 underwent splenectomy/pancreatectomy (PS), and 1 755 underwent gastrectomy alone. The PS and S groups were associated with a higher percentage of proximal tumors and TG, higher T stage, higher N stage, and worse histologic grade. There were more severe post-operative

complications in the PS and S groups over the gastrectomy alone group, with a higher rate of pancreatic fistula, intra-abdominal abscess, and anastomotic leak. There was also a higher rate of local recurrence in the S and PS groups. When analyzed by UICC stage; however, no difference in 5-year survival for stage II, III, or IV tumors was detected. Gastrectomy alone was found to have higher 5-year survival for T2 tumors; however, for T3 and T4 tumors, there was no difference. Conclusions from this study were that long-term survival is not affected by splenectomy or pancreatectomy and these procedures should not be performed solely to aid in lymph node dissection. The spleen should only be resected when there are clearly positive lymph nodes in the splenic hilum and around the splenic artery, and the pancreas should only be resected when there is direct invasion of tumor.

Resection of adjacent organs for local invasion was advocated in the early Japanese literature^[10]; however, this practice also has been analyzed extensively in the past decade. Shchepotin *et al*^[19] reviewed 353 patients with T4 gastric cancer who underwent gastrectomy combined with resection of adjacent organs. Of these patients, 89% had histologically confirmed invasion. TG was performed in 32.9% of patients and SG in 67.1%. The extent of lymph node dissection was the same in all patients, consisting of dissection of all perigastric nodes, nodes along the celiac axis, hepatic artery, and proximal splenic artery. The transverse colon was resected in 45%, the tail of the pancreas and spleen in 42.5%, the left hepatic lobe in 28.5% and the head of the pancreas in 10.5%. The complication rate was 31.2%, and mortality rate was 13.6%. Combined 5-year survival was 25%; however, when this was broken down into node positive or node negative T4 lesions, 5-year survival changed significantly. Node negative T4 patients had a 37% 5-year survival, whereas node positive T4 patients had only a 15% survival. From these data, the authors continued to advocate extended resection of adjacent organs in the resection of gastric cancer for cure.

Martin *et al*^[20] showed that extended organ resection could be performed with acceptable morbidity (4%) and 5-year survival (32%). Two hundred and sixty-eight patients with locally advanced gastric cancer underwent gastrectomy with adjacent organ resection and D2 lymphadenectomy. The type of gastrectomy was variable; however, there was a higher incidence of adjacent organ resection in the TG group. Spleen alone was the most common resection ($n=123$), followed by spleen/pancreas ($n=38$), spleen/colon ($n=18$), pancreas alone ($n=12$), and colon alone ($n=16$). As would be expected, there was a higher percentage of T3/T4 tumors and N2/N3 lymph node metastases in the organ resection group. Perioperative mortality was not significantly different, 3.6% in the gastrectomy group and 3.7% in the gastrectomy with organ resection group. There was a higher rate of recurrence in the gastrectomy with organ resection group (52% *vs* 42% at 24 mo follow-up), and significantly lower median survival (32 mo *vs* 63 mo) on univariate analysis. On multivariate analysis, however, only T-stage, N-stage and overall stage were predictors of survival. The authors concluded that resection of adjacent organs is important

to achieve an R0 resection and can be done with minimal morbidity; however, careful selection of clinically T4 tumors should be performed to limit unnecessary organ resection in early stage gastric cancer.

Resection of adjacent organs in conjunction with gastrectomy can increase survival with minimal additional morbidity in a highly selected patient population.

POSITIVE MARGINS

Patients with recurrence after resection of gastric cancer uniformly have a rapid decline. The risk factors for recurrence, and the rate and pattern of recurrence has been examined. In a recent retrospective review of 367 patients who underwent an R0 resection with subsequent recurrence, the rate and pattern of recurrence was examined^[21]. The majority of the patients (65%) had T3 lesions, 46% had N1 nodal disease, and 54% were AJCC stage III. The type of gastric resection was highly variable, with the highest percent being esophago-proximal resections (45%). The extent of lymphadenectomy was also variable, with the majority being D2 (68%). The rate of locoregional, peritoneal, and distant metastases was 54%, 51%, and 29% respectively, with significant overlap of these three. Seventy-nine percent had recurred within two years and 94% by four years. The median time to death from diagnosis of recurrence was 6 mo. The pattern of recurrence was not associated with survival time; however, T stage, N stage, age and presentation with symptoms were all predictors of decreased survival. Advanced T stage, distal location, diffuse Lauren's subtype, and female gender were significantly associated with peritoneal metastases. Proximal location and male gender were associated with locoregional recurrence. Proximal location, early T stage, and intestinal Lauren's subtype were associated with distal recurrence. There was no significant pattern of recurrence associated with overall stage, extent of lymphadenectomy, nodal status, or extension of resection to adjacent organs.

There is no surgical therapy for recurrent gastric cancer; however, the question of re-excision for positive margins at primary resection has been raised. Survival for patients with microscopically positive margins has been shown to be significantly shorter than those with clear margins^[25]. In a recent review of 259 patients who underwent gastrectomy with curative intent, 22 had microscopically positive resection line margins^[22]. Positive margins were associated with tumor location and differentiation. There was a significantly lower 5-year survival for the patients with positive margins (18% *vs* 45%); however, when this was stratified for lymph node status, the patients with positive lymph nodes had a significantly lower survival. The authors concluded that, given the very poor prognosis associated with positive margins, re-laparotomy may be justified in those patients with node-negative disease.

PALLIATIVE SURGERY FOR GASTRIC CANCER

Despite improved clinical outcomes associated with earlier diagnosis, more accurate staging, and decreased surgical morbidity and mortality, the overall prognosis of gastric

cancer remains poor because many patients are incurable at presentation. A complete R0 resection remains the most powerful indicator of survival, but is obtained in only 50% of those presenting for resection of a primary gastric cancer^[20,23]. For those patients who present with stage IV disease, cure measured by 5-year survival is exceedingly rare and is not a realistic treatment goal^[2,24-32]. Because of the low cure rate and the advanced stage at which many patients present, palliative strategies and symptom management remain an essential component in the total care of the patient with gastric cancer^[33].

Palliation of symptoms caused by any advanced cancer demands the highest level of surgical judgment. Although an important part of the surgical decision making process requires consideration of risk in terms of treatment-related toxicity, morbidity, and mortality, attention to this element should not be the predominant factor in making decisions about palliative therapy. Decisions are made best on endpoints such as the probability and durability of symptom resolution, the impact on overall QOL, and pain control. Deliberations must consider the medical condition and performance status of the patient, the extent and prognosis of the cancer, the availability and success of non-surgical management, cost effectiveness and the individual patient's quality and expectancy of life. Knowledge about the need to repeat a specific therapy or requirements to manage additional symptoms can give further information about the potential for symptom-free survival and what additional care will be needed. Therapy for symptoms must remain flexible and individualized to continually meet the patient's unique and ever changing needs^[34,36]. Surgical palliation of advanced gastric cancer may include resection or bypass, alone or in combination with endoscopic or percutaneous interventions. Such interventions have been proposed not only to improve symptom control, but also to eliminate potential complications (bleeding, obstruction, pain, perforation, and debilitating ascites) caused by the primary tumor^[37,38].

In 1958, Lawrence and McNeer demonstrated that palliative gastric resections effectively relieve symptoms in patients with incurable gastric cancer. Because of high rates of associated perioperative morbidity and mortality and the brief period of anticipated survival, however, the authors suggested that a TG in patients with incurable gastric cancer was rarely a worthwhile palliative procedure^[39]. This conclusion was supported by Remine in 1979 who also suggested that TG was not a satisfactory palliative operation^[40]. Later series, however, showed improved symptom relief with gastrectomy compared to gastroenterostomy, without increasing complication rates^[41,42]. Others have based their support for palliative gastric resections primarily on improved survival data and have proposed that it should be performed whenever technically possible^[43,44]. Because of decreasing perioperative complications, some authors now suggest that total palliative gastrectomy and esophagogastrectomy is justified in selected patients^[45-49].

The effective and appropriate use of palliative surgical interventions in patients with gastric cancer remains controversial. Recommendations from the literature are contradictory and often based on the retrospective

evaluation of suboptimal data. A highly variable and imprecise understanding of the goals and indications of palliative surgery, poorly defined patient groups, and a reliance on inappropriate endpoints contributes to this confusion. The designation of patients as "palliative" is commonly based on the extent of disease (ranging from gross disease at operation to postoperative margin status) rather than a sound definition encompassing factors associated with good palliative therapy. Even though the value of a palliative procedure should be judged by its ability to control symptoms, reports often fail to utilize validated QOL or pain assessment instruments and rarely consider the durability of potential palliative benefits^[25]. These factors limit useful interpretation of most prior studies on palliative procedures for gastric cancer. The impact of such deficiencies in the literature has been demonstrated by a recent analysis of the Memorial Sloan-Kettering Gastric Cancer Database. Important differences among the patients undergoing a non-curative gastric cancer were demonstrated when a sound and reproducible definition of palliative surgery was applied. Significant differences between overall survival, primary tumor sites, staging, degrees of nodal and metastatic disease, and the types of procedures performed support the differentiation between palliative and non-palliative designations^[49].

Conclusions over the effectiveness of palliative operations in the gastric cancer literature are often based, incorrectly, on incremental survival differences. Caution must be taken when evaluating survival data in patients following a palliative intervention. Palliative care ideally selects treatment that will maximize QOL and minimize complications. Consideration of anticipated survival helps to define a period during which the requirements of effective symptom control must be met and may be useful when considering the risk-benefit ratio for an individual patient^[50,51]. Although increased survival may be a secondary goal of a palliative procedure, it is inappropriate to select a palliative procedure solely based on improved duration of survival^[36]. Based on patients grouped by extent of disease rather than palliative intent, The Dutch Gastric Cancer Group has suggested that differences in overall survival following "palliative" gastric resections may be beneficial in patients with tumor load restricted to one metastatic site^[52]. Such conclusions about the value of palliative gastrectomy are premature because they fail to consider the associated risks, benefits, and expected durability of the procedure^[33,49].

To compare the impact of potentially achievable gains associated with palliative surgery, non-curative gastrectomies were analyzed with a partitioned survival analysis^[33,53]. The technique, which has been generally used to evaluate chemotherapy trials, analyzes treatment by defining relevant clinical health states and comparing their duration with regard to treatment, toxicity and relapse. The analysis provided by this methodology is well suited to evaluate surgical palliation, especially in conditions such as gastric cancer, in which treatment-related toxicity plays such an important role in both the literature and in clinical decision-making^[33,54]. Palliative gastric resections previously have been associated with considerable operative morbidity (54%) and mortality (6%)^[49]. Duration in the time without

symptoms or toxicity (TWiST) state was found to be significantly decreased (8.5 mo *vs* 2.1 mo, $P=0.04$) in patients experiencing a major postoperative complication (requiring invasive interventions, unplanned ICU admission, or permanent disability). Complications have a considerable influence on the patients' limited survival by not only increasing time in a hospitalized setting but also by diminishing the QOL. Concerns about potential complications such as bleeding, obstruction or perforation have led some to propose prophylactic gastric resections in patients with advanced, asymptomatic gastric cancer. Sarela *et al* have shown in patients with known metastatic disease identified on laparoscopy; however, that complications requiring emergency or urgent palliative operations were rare in patients not receiving a prophylactic resection for incurable gastric cancer^[55].

Since palliative gastrectomies are associated with significant perioperative morbidity and mortality, the authors recommend deliberate palliative resection only in carefully selected patients with severe symptoms^[34,50,54,55].

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REVIEW

Gene therapy for gastric cancer: Is it promising?

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Abstract

Gastric cancer is one of the most common tumors worldwide. The therapeutic outcome of conventional therapies is inefficient. Thus, new therapeutic strategies are urgently needed. Gene therapy is a promising molecular alternative in the treatment of gastric cancer, including the replacement of defective tumor suppressor genes, the inactivation of oncogenes, the introduction of suicide genes, genetic immunotherapy, anti-angiogenic gene therapy, and virotherapy. Improved molecular biological techniques and a better understanding of gastric carcinogenesis have allowed us to validate a variety of genes as molecular targets for gene therapy. This review provides an update of the new developments in cancer gene therapy, new principles, techniques, strategies and vector systems, and shows how they may be applied in the treatment of gastric cancer.

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INTRODUCTION

Gastric cancer is the fourth most common malignancy worldwide with an estimated 934 000 new cases that was reported in 2002 and the second most common cause of death from cancer (700 000 deaths annually). Almost two-thirds of the cases occur in developing countries and 42% in China alone^[1]. The prognosis of gastric cancer is poor with an estimated relative 5-year survival rate of less than 20%^[2]. The efficacy of current therapeutic approaches such as surgery, hormone, radio- and chemotherapy is

limited. Thus, new therapeutic approaches are urgently needed. Cancer is an acquired genetic disease developing in a multi-step process. Mutations of genes related to growth control, apoptosis, invasion and metastasis form the molecular genetic basis of malignant transformation and tumor progression^[3]. The characterization of dysregulated genes like the tumor suppressor p53, which are critical for carcinogenesis, and a better understanding of the molecular basis for tumor-host interaction led to significant progress in the development of new therapeutic agents. More than 15 years ago, gene therapy emerged as a new therapeutic approach and has meanwhile become an important strategy in cancer treatment. Cancer is by far the most frequent of all indications addressed by gene therapy (60% of all clinical trials^[3]), underlining the expectations raised by this new therapeutic option. The original concept of cancer gene therapy was further developed into two branches as a result of different strategies of therapeutic benefit: molecular cancer therapy and virotherapy. Molecular cancer therapy can be defined as a therapeutic technique, which aims at the introduction of nucleic acids into cancer patients' cells in order to modulate the gene expression profile of the target cells and thereby eradicate the tumor^[4]. In contrast, virotherapy is a new concept of gene therapy that uses replication-competent oncolytic viral vectors (OVV) with viro-oncolytic potency for targeted tumor cell destruction^[5].

There are different ways to modulate tumor growth by gene therapeutic strategies. These include direct destruction of tumor cells, inhibition of tumor angiogenesis and tumor cell spread and activation of the host immune response against the tumor. Although all these approaches showed promising anti-tumor effects in pre-clinical investigations, clinical trials have often been disappointing, since they demonstrated only slight therapeutic benefit. Thus, a major breakthrough is still needed in gene therapy of cancer. Nevertheless, clinical trials proved the relative safety of human cancer gene therapy. The application of vectors and the expression of transgenes are generally well tolerated and the low risk of severe side effects seems to be calculable.

The main problem of the relative inefficiency of cancer gene therapy continues to be the low *in vivo* efficiency of gene delivery into the target tumor cells, leading to low expression of therapeutic genes and thus limited curative effects. Several factors seem to be responsible for this, among them the presence of anatomic barriers inhibiting the efficient transfer of vectors from circulation to target cells and the low expression of vector-specific target receptors on the cancer cells causing reduced

cellular uptake of viral vectors. Moreover, immunological responses of the host against the vectors and the rapid clearance of vectors from circulation after intravascular administration may be important factors preventing efficient gene transfer^[6-8]. In the last few years, great efforts have been made to overcome these limitations. Especially the development of OVV^[9] and vectors with enhanced tumor-specific targeting^[10,11] together with improved vector application protocols have led to a significant enhancement of vector-mediated gene delivery. Furthermore, the use of new powerful molecular techniques like RNA interference (RNAi)^[12] and the detection of new target genes are hopeful signs for improving human cancer by gene therapy. In the present paper, we review new trends in gene therapy and update their application in gastric cancer.

TUMOR SUPPRESSOR GENES

The most obvious way to target growth regulation in cancer cells is to introduce tumor suppressors that may be inactivated in tumors. The replacement of p53, which is the most commonly inactivated tumor suppressor and mutated in about 60% of human gastric cancers, has emerged as an attractive treatment option, both alone and combined with conventional chemotherapy^[13,14]. Introduction of the p53 gene via a recombinant adenovirus has been shown to inhibit the growth of gastric cancer cells with mutated p53 *in vitro* and *in vivo*^[15,16]. The pro-apoptotic function of p53 depends on the transactivation of genes such as Bax, Apaf-1, Fas, and PTEN, whose own expression or activity may be abnormal in tumor cells^[17]. Consequently, the Bax gene may serve as a good alternative to p53 for cancer gene therapy, not only because it has been shown to kill cancer cells irrespective of their p53 status, but also because it may increase their sensitivity to other anti-tumor treatments^[18]. Moreover, the adenoviral expression of the initiator caspase-8 leads to the selective induction of apoptosis in detached gastric tumor cells *in vitro* and *in vivo*, thus displaying anti-metastatic potential in gastric cancer^[19]. All these signaling molecules work through a common pathway involving activation of the effector caspase 3. Thus, recombinant expression of caspase 3 leads to the induction of apoptosis in gastric cancer cells^[20]. Moreover, the introduction of wild-type p16INK4A, another tumor suppressor and cell cycle regulator in gastric cancer cells harboring a p16 mutation, may also be a feasible approach to efficient tumor growth control and chemosensitization^[21]. Finally, the replacement of Fhit, a tumor suppressor often inactivated in gastric cancer, decreases sensitivity to carcinogens and induces apoptosis in gastric tumor cells *in vivo*. Therefore, restoring Fhit expression by viral transduction may be a promising strategy for both the prevention and therapy of gastric tumors^[22].

SUICIDE GENES

This strategy relies on the conversion of non-toxic substances (prodrugs) into physiologically active agents by means of non-mammalian enzymes. These suicide enzymes are over-expressed in neoplastic cells as a

result of successful transfection with their genes^[23]. The most widely used suicide gene/prodrug system is the herpes simplex virus (HSV) thymidine kinase (HSV-tk)/ganciclovir (GCV) system that can convert the prodrug GCV into phosphorylated GCV. The phosphorylated GCV inhibits cellular DNA synthesis and leads to the killing of cancer cells via apoptotic and non-apoptotic mechanisms^[24,25]. One of the powerful features in these systems is the “bystander effect”, the mechanism by which the toxic metabolites are transferred from transduced cells to neighboring cancer cells via gap junctions or apoptotic vesicles. The bystander effect drastically enhances the tumor-killing capacity of the HSV-tk/GCV system^[26,27]. Several studies were undertaken to evaluate the potential of suicide gene therapy in gastric cancer. In alpha-fetoprotein (AFP)-producing gastric tumors, the adenovirus-mediated expression of HSV-tk by an AFP enhancer/promoter element selectively eliminated AFP-positive, but not AFP-negative cell lines when treated with ganciclovir^[28]. This approach may be a promising tumor-selective treatment option for AFP-positive gastric tumors with a very poor prognosis. A similar approach involves the expression of recombinant *E. coli* cytosine deaminase (CD) in gastric cancer cells together with the administration of 5-fluorocytosine (5-FC). 5-FC is given orally and converted to 5-fluorouracil in the tumor cells expressing CD. In attempts to increase the specificity of suicide gene therapy, CD expressed from gastric cancer cell-specific promoters SEL1L and TP1 was shown to cause efficient cytotoxic effects in combination with 5-FC^[29]. An earlier attempt with tumor-specific and more efficient CD/5-FC gene therapy was carried out using the Cre/loxP regulation system. Ueda *et al.*^[30] constructed an adenovector-expressing Cre recombinase from a carcinoembryonic antigen (CEA) promoter and a second vector expressing CD under the control of the CAG promoter. The double infection with both vectors rendered CEA-producing gastric cancer cells 13 times more sensitive to 5-FC than the single infection with a vector expressing CD from the CEA promoter. Consequently, anti-tumor efficacy *in vivo* was also significantly enhanced by using the Cre/loxP system compared to the single infection with the vector directly expressing CD under the control of the CEA promoter. Finally, recombinant expression of the bacterial enzyme nitroimidazole reductase gene together with the administration of the prodrug CB1954 was evaluated in a phase I and pharmacokinetic study with the intention of treating gastric cancer^[31].

ANTI-ANGIOGENESIS GENE THERAPY

Tumor angiogenesis plays an important role in the growth of solid tumors and the formation of metastases. Angiogenesis is a multi-level process including endothelial cell proliferation, migration, basement membrane degradation, and lumen reorganization. It is stimulated by several factors secreted from both host and tumor cells. The principal growth factors driving angiogenesis include, among others, the vascular endothelial growth factor (VEGF), the basic fibroblast growth factor, and the hepatocyte growth factor (HGF)^[32]. Thus, there are various

potential targets for anti-angiogenic cancer gene therapy. In contrast to other genetic treatments, anti-angiogenic gene therapy does not necessarily require direct and selective transduction of target genes into cancer cells, but rather transduction around the tumor to create an anti-angiogenic environment^[33]. This advantage helps to overcome the limitations of the currently available vector systems, which often lack adequate transduction efficiency in cancer cells. Several studies were undertaken to evaluate the potential of anti-angiogenic gene therapy in gastric cancer. One study demonstrated that, if expressed from adenovector-transduced peritoneal mesothelial cells, the soluble VEGF receptor sFlt-1 is able to inhibit the peritoneal dissemination of gastric cancer *in vivo* and consequently prolong the survival of treated animals^[33]. Another study evaluated the therapeutic efficacy of the HGF antagonist NK4, which is known for its inhibitory effects on several angiogenic pathways. Application of an NK4-expressing adenovector inhibited the formation of both peritoneal metastases and intra-tumor vessels in gastric cancer *in vivo*^[34]. New potential targets for anti-angiogenic gene therapy of gastric cancer were recently discovered. Meng *et al*^[35] and Xue *et al*^[36] showed that silencing Raf-1 and Rac1 GTPase, which are critical factors in hypoxia-induced gene activation of several angiogenesis factors, results in downregulation of the angiogenesis-promoting factors VEGF and Hif-1 α and upregulation of the tumor suppressors and angiogenesis inhibitors p53 and VHL. Furthermore, downregulation of Raf-1 and Rac1 GTPase leads to tumor cell apoptosis and significantly inhibits cell proliferation. Similarly, Stoeltzing *et al*^[37] showed that direct suppression of Hif-1 α resulted in decreased secretion of VEGF, thereby impairing tumor growth, angiogenesis and vessel maturation *in vivo*.

GENETIC IMMUNOTHERAPY

Genetic immunotherapy aims at improving the host's immune response to a particular tumor and is currently one of the most promising gene therapeutic options for cancer. The function of the immune system is very complex and its activation in gene therapeutic settings can be achieved by employing different strategies^[38]. One of the most common strategies in immunotherapy of cancer is the use of mediators of the immune system. Among them, IL-2, IL-12, INF- γ , GM-CSF and TNF- α have raised special attention and several trials have proved their efficacy in cancer gene therapy^[39-42]. New developments indicate further improvement of the benefit, if cytokine therapy is combined or used with other gene therapeutic options. For example, synergistic anti-tumor effects were achieved by simultaneous expression of IL-2 and INF- γ ^[40] or by combining an oncolytic adenovirus (oAdV) with IL-12 immunotherapy^[43].

Based on this knowledge, studies were carried out in order to prove the efficacy of immunotherapy in combination with other gene therapeutic strategies in gastric cancer. Zhang *et al*^[44-46] evaluated the anti-tumor effects of the HSV-tk/GCV system together with the expression of recombinant IL-2 or TNF- α in gastric cancer. In contrast to their disappointing results *in vitro*^[46],

they observed enhanced anti-tumor effects by HSV-tk/GCV suicide gene therapy combined with recombinant TNF- α expression *in vivo*^[44]. Using a similar protocol, another group found strongly enhanced anti-tumor effects after coexpression of IL-2, GM-CSF and HSV-tk/GCV in a gastric cancer model *in vivo*^[47]. These results strongly indicate the potential impact of combined cytotoxic and immunomodulatory gene therapy in gastric cancer. Other immunotherapeutics also demonstrated their potential efficacy in gastric cancer. For example, it was shown that the expression of recombinant intercellular adhesion molecule (ICAM)-2 prolonged the survival of mice with peritoneal metastases of gastric cancer^[48]. Meng *et al*^[49] tested the recently discovered gastric carcinoma-specific tumor-associated antigen MG7-Ag in a *Salomonella typhimurium* vaccine against gastric cancer. In detail, they constructed a recombinant gene vaccine consisting of the MG7-Ag mimotope fused with HBcAg, a protein from HBV enhancing the immunogenicity of its antigens. Oral application of the vaccine *in vivo* led to increased formation of MG7-Ag antibodies, reduced average tumor weight compared to the controls and prevented tumor growth in one of five immunized mice, thereby indicating some protective effects of the vaccine^[50].

GENE SILENCING APPROACHES

Inappropriately expressed genes are a major cause of uncontrolled cell growth. Thus, the specific downregulation of (onco)gene expression leading to tumor growth inhibition is a promising approach in cancer gene therapy^[51]. Several years ago, double-strand RNA molecules homologous to the sequence of the target gene were shown to induce post-transcriptional gene silencing (PTGS) in a sequence-specific manner. This mechanism was designated as RNAi^[52]. The process of PTGS is initiated by small interfering (si) RNA molecules, which have a length of 21-23 nucleotides^[12]. In mammalian cells, siRNAs are incorporated into a large protein complex, the RNA-induced silencing complex, leading to precise degradation of complementary mRNA targets^[53]. Due to its extraordinary efficiency, target gene specificity and simplicity of construction, siRNA technology has gained considerable attention as a new tool for gene knockdown and, hence, therapeutic use in cancer gene therapy. Chemically synthesized or *in vitro*-transcribed siRNAs are widely used for *in vitro* anti-cancer studies, while their use *in vivo* revealed several problems. Major limitations *in vivo* are the generally low transduction efficiency and short half-life. Furthermore, synthetic siRNAs preferentially transduce the liver after systemic application^[54], rendering them useless for systemic cancer gene therapy. These obstacles may be overcome by the expression of siRNA from viral vectors. Currently, adenoviral, retroviral and adeno-associated virus vectors have been shown to efficiently express siRNAs resulting in strong downregulation of the target gene^[55-57]. In this setting, vector-based expression systems were further developed, enabling tissue-specific and inducible siRNA expression by the use of tissue specific promoters^[58] and pharmacologically regulated gene expression systems^[55].

Furthermore, siRNA expressed from viral vectors seems to be more stable than synthetic siRNA^[59]. Several studies were undertaken to evaluate siRNA technology in gastric cancer. Hong *et al.*^[60] constructed a eukaryotic vector expressing siRNA against new zinc ribbon (ZNRD1) gene, which promotes a multi-drug resistant phenotype in gastric cancer through the upregulation of permeability-glycoprotein. After transfection of a ZNRD1 siRNA, a dramatic reduction of ZNRD1 was observed accompanied by a significantly enhanced sensitivity to vincristine, adriamycin and etoposide. Further studies proved the high efficiency of siRNA-mediated gene silencing in gastric cancer cells *in vitro*^[35,59,61-63]. Continuous development of siRNA technology warrants further investigations of its future therapeutic use in gastric cancer *in vivo*.

Further approaches for the downregulation of tumor genes in gastric cancer, including anti-sense-RNA^[64], anti-sense oligonucleotides^[65,66], ribozymes^[67], and dominant negative forms of tumor proteins^[37,68], have also been investigated and may be of potential clinical value in the gene therapy of gastric cancer. While anti-sense strategies preferentially aim at blocking the translation of a target mRNA by complementary binding to its specific mRNA, dominant negative mutant alleles compete with their endogenous homologs for binding in a protein complex, leading to the inhibition of protein function. For example, the insulin-like growth factor (IGF) I receptor is involved in carcinogenesis and proliferation. Its blockade by adenovector-mediated expression of a truncated dominant negative IGF was shown to sensitize gastric tumor cells for chemotherapy and to suppress their peritoneal dissemination *in vivo*^[68]. In another study, Kim *et al.*^[66] showed that downregulation of anti-apoptotic protein bcl-2 by administering bcl-2 anti-sense oligonucleotides significantly increased the sensitivity of gastric cancer to chemotherapeutics *in vivo*.

VIROTHErapy

The limited efficiency of replication-deficient viral vectors to transduce cancer cells and express effector genes *in vivo* led to the development of a new vector generation called OVV. In contrast to replication-deficient viral vectors, the primary replication cycle of OVV causes viro-oncolysis of initially infected tumor cells, resulting in the release of progeny virions followed by the infection of adjacent cells and the infection and destruction of further tumor mass^[69]. Thus, OVV are intended to ultimately destroy a tumor although only a small percentage of tumor cells was initially infected. Furthermore, progeny virions can spread systemically by circulation^[70] and infect tumor cells remote from the primary replication site of OVV, thus enhancing the potential therapeutic efficacy in metastatic cancer.

The restriction of OVV replication to cancer cells is a central concern of OVV development. This aim has been achieved by genetic engineering of viral vector genomes (e.g. in herpes- and adenoviruses) either by driving of viral genes essential for virus replication by tumor-specific promoters^[71,72] or by inserting mutations into viral genes that abolish their function for viral replication in normal cells but not in tumor cells^[73]. Other OVV with inherent

oncolytic potency acquire tumor-selective replication competence through defects or dysregulation of cellular genes in cancer cells (e.g. Newcastle virus and vesicular stomatitis virus)^[74,75].

Several studies have demonstrated that replication of OVV is 100- to 1 000-fold attenuated in normal cells compared to cancer cells. As shown in oAdVs, OVV safety can be further increased by pharmacological regulation of viral replication, using the rapamycin^[76] or the Tet-On gene expression system^[77,78] to regulate adenoviral E1A. This now opens the door to permanent external control of OVV during the treatment of patients. Various genetically engineered OVV and viruses with inherent oncolytic properties have recently been explored as anti-cancer agents, among them adenovirus^[9,79,80], HSV^[81-83], retroviruses^[84], vaccinia virus^[41], autonomous rodent parvovirus^[85], vesicular stomatitis virus^[86], Newcastle virus^[87], and reoviruses^[88-89]. Of these, HSV and adenovirus are the most widely studied ones. ONYX-015 was the first tested oAdV and is to date the most commonly used oAdV in clinical trials. Deletion of the adenoviral E1B-55kD enables the replication of ONYX-015 in cells with a defective p53 pathway and minimizes its replication in cells with a functionally active p53 pathway^[9]. Thus, ONYX-015 is unable to replicate in normal cells, but strongly replicates in cancer cells. Several clinical trials have demonstrated the efficacy of ONYX-015 in patients with cancer. Strongest anti-tumor responses were observed in patients with squamous cell cancer of the head and neck^[90,91], but responses to hepatocellular carcinoma^[92], hepatobiliary tumors^[93], and advanced pancreatic cancer^[94] were reported, whereas no response was observed in patients with advanced ovarian cancer^[95]. Two phase I/II clinical trials have provided evidence for the efficacy of ONYX-015 in metastatic gastrointestinal cancer^[96,97]. Reid *et al.*^[96] administered ONYX-015 by hepatic artery infusion combined with 5-fluorouracil and leucovorin in 27 patients with both primary gastrointestinal carcinoma and liver metastases. The treatment was well tolerated showing only mild or moderate flu-like symptoms, including fever, myalgia, asthenia and/or chills. Virus replication was demonstrated and three partial responses, four minor responses and nine stable diseases were documented as therapeutic outcome. In another study, patients with advanced sarcomas, among them patients with gastrointestinal stromal tumors, were given an intratumoral injection of ONYX-015 combined with MAP chemotherapy. The treatment was well tolerated and there was no significant toxicity. One of the six patients treated showed a partial response with an approximately 70% reduction of tumor size, and in four patients the disease stabilized^[97]. Other oncolytic viruses like HSV, Newcastle virus and vaccinia virus also demonstrated their viro-oncolytic efficacy in clinical trials with cancer patients^[41,98,99]. On the other hand, the studies revealed therapeutic limitations of the currently available OVV. Often, only a minority of patients shows a response, which is only partial and transient in most of the cases^[5,97]. Obviously, there are several major limitations to the therapeutic potential of OVV. The key problems are low infectivity, replication rate and cytolytic activity of OVV.

To overcome these limitations, measures have therefore been taken to further develop OVV. The low transduction efficiency of oAdV due to low coxsackie-adenovirus receptor (CAR) expression can be enhanced by modifying the fiber proteins. This can be achieved by adding foreign peptides to the HI loop or the C-terminus of the fiber knob^[100,101] or by substituting fibers of adenoviral 2 and 5 with fibers derived from other adenoviruses, which bind to receptor molecules other than CAR^[102,103]. These strategies seem to be promising for the treatment of gastric cancer as well, since gastric cancer cells express low amounts of CAR, making it resistant to adenoviral infection^[100]. Recently published data demonstrate that oAdV with RGD motif in the HI-loop of the fiber-knob region or replacing its adenovirus type 5 knob by an adenovirus type 3 knob has a stronger anti-tumor effect than unmodified oAdV in a gastric cancer model *in vivo*^[100]. Another study investigated re-targeting a doubly-ablated adenovector to the epithelial cell adhesion molecule (EpcAM) by introducing a bi-specific single-chain antibody to EpcAM. EpcAM is highly expressed in gastric cancer but not in gastric epithelium. Consequently, the vector was highly selective for primary gastric tumors, while transduction of normal gastric epithelium and liver was low^[104].

Another way to improve the efficacy of OVV is combining OVV treatment with conventional and other gene therapeutic strategies. Preclinical and clinical data demonstrate that OVV-induced tumor cell killing can be strongly enhanced by the expression of therapeutic transgenes from OVV like anti-angiogenic factors, suicide genes, or tumor suppressor genes and simultaneous treatment with conventional chemo- and radiotherapy^[43,96,105-110].

PROSPECTS

Gene therapy has become a generally accepted new therapeutic tool in the treatment of cancer. More and more cancer patients profit from its use due to the progress made in the development of vector systems and gene therapeutic strategies. Thus, cancer gene therapy will increase its importance as a therapeutic tool even though many problems still need to be solved. One of the most important issues affecting the possible clinical application of gene therapy is the need to ensure the highest possible safety levels. Many clinical investigations have demonstrated that the currently available vector systems are well tolerated and side effects are acceptable. However, the use of retroviral vectors is discussed controversially, since 3 of 11 children with X-linked severe combined immunodeficiency, who were treated with a retrovirus, developed uncontrolled T-lymphocyte proliferation in a French gene therapy trial.

The major problem of cancer gene therapy that still remains is the relatively poor therapeutic outcome. This problem is not restricted to a specific tumor entity, but is rather a general problem. There may be many reasons for this, but it is widely agreed that this is mainly due to the relative resistance of cancer cells to introduce foreign material combined with low transgene expression *in vivo*. Thus, improved vector systems and application protocols

will continue to be the biggest issues to be dealt with in cancer gene therapy in the next few years. However, important progress to overcome these limitations has already been made by the development of OVVs and vectors with increased tumor cell tropism.

Great progress has also been made in the development of gene therapeutic strategies in gastric cancer. New vector systems as well as the evaluation of new target genes and gene therapeutic strategies have substantially improved the chances for successful treatment of gastric cancer by gene therapy. The next challenge will be to test the results gained thus far in clinical studies.

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BASIC RESEARCH

Effect of nuclear factor kappa B on intercellular adhesion molecule-1 expression and neutrophil infiltration in lung injury induced by intestinal ischemia/reperfusion in rats

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CONCLUSION: The activation of NF- κ B plays an important role in the pathogenesis of lung injury induced by intestinal I/R through upregulating the neutrophil infiltration and lung ICAM-1 expression. PDTC as an inhibitor of NF- κ B can prevent lung injury induced by intestinal I/R through inhibiting the activity of NF- κ B.

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Key words: Lung injury; Intestinal ischemia/reperfusion; NF- κ B; ICAM-1; Neutrophil infiltration

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Abstract

AIM: To investigate the role of nuclear factor kappa B (NF- κ B) in the pathogenesis of lung injury induced by intestinal ischemia/reperfusion (I/R), and its effect on intercellular adhesion molecule-1 (ICAM-1) expression and neutrophil infiltration.

METHODS: Twenty-four Wistar rats were divided randomly into control, I/R and pyrrolidine dithiocarbamate (PDTC) treatment groups, $n = 8$ in each. I/R group and PDTC treatment group received superior mesenteric artery (SMA) occluding for 1 h and reperfusion for 2 h. PDTC group was administrated with intraperitoneal injection of 2% 100 mg/kg PDTC 1 h before surgery. Lung histology and bronchia alveolus lung fluid (BALF) protein were assayed. Serum IL-6, lung malondialdehyde (MDA) and myeloperoxidase (MPO) as well as the expression level of NF- κ B and ICAM-1 were measured.

RESULTS: Lung injury induced by intestinal I/R, was characterized by edema, hemorrhage and neutrophil infiltration as well as by the significant rising of BALF protein. Compared to control group, the levels of serum IL-6 and lung MDA and MPO increased significantly in I/R group ($P = 0.001$). Strong positive expression of NF- κ B p65 and ICAM-1 was observed. After the administration of PDTC, the level of serum IL-6, lung MDA and MPO as well as NF- κ B and ICAM-1 decreased significantly ($P < 0.05$) when compared to I/R group.

INTRODUCTION

Intestinal I/R is not necessarily limited to the intestine itself, but involves severe destruction of distant tissue. It is known that intestinal I/R is an important event in the pathogenesis of multi-system organ failure syndrome, especially acute respiratory distress syndrome which is the leading cause of death in critically ill patients^[1-4]. The mechanism of lung injury induced by intestinal I/R is complex. Proinflammatory cytokines and chemokines exert their effects via a direct toxic action on target cells^[5], ICAM-1 and neutrophil infiltration play an important role in lung injury induced by intestinal I/R^[6]. However, recent studies showed that these proinflammatory mediators play a role in gene induction^[7-9]. NF- κ B upregulates most of these mediators^[10-12], but it is not known how NF- κ B is activated and ICAM-1 is expressed in lung injury induced by intestinal ischemia.

This study was to evaluate the role of NF- κ B in the pathogenesis of lung injury induced by intestinal I/R and the effect of pyrrolidine dithiocarbamate (PDTC) on lung neutrophil infiltration and expression of ICAM-1.

MATERIALS AND METHODS

Animals

Male Wistar rats (From Animal Center of Dalian Medical

University, Dalian, China) weighing 200-240 g were used in this study. All rats had free access to standard laboratory chow and water in accordance with institutional animal care policies.

Experimental design

The rats were anesthetized with intraperitoneal administration of 10% chloral hydrate, 350 mg/kg, laparotomized and randomly divided into three experimental groups ($n=8$ in each): sham operation group (control group) undergoing full surgical preparation including isolation of SMA without occlusion; I/R group: ischemia was induced for 1 h and reperfusion for 2 h after SMA was isolated and ischemia was occluded^[13]; PDTC treatment group undergoing full surgical preparation including isolation of SMA with intraperitoneal administration of 2% PDTC 100 mg/kg 1 h before the operation. The rats in control and I/R groups were treated with an equal volume of normal saline solution. All animals were killed after 2 h of reperfusion. Blood samples were obtained for analysis. Lung tissues were harvested immediately for detection.

Lung histology and bronchia alveolus lung fluid (BALF) assay

The harvested right middle lobe of the lung was fixed in 40 g/L formaldehyde. After being embedded in paraffin, 4- μ m-thick sections were stained with hematoxylin and eosin for light microscopy. Pathological injury score was evaluated according to Chiu's method^[14]. BALF was collected according to the process of Cox^[15] and centrifuged at 1 000 r/min for 10 min. The protein in the supernatant was measured using assay kit (Nanjing Jincheng Corp., China) following the manufacturer's instructions and expressed as g/L.

Serum IL-6 assay

The serum level of IL-6 was determined using an RIA kit (Radioimmunity Institute of PLA General Hospital, Beijing, China) according to the manufacturer's instructions and expressed as ng/L.

Lung MDA and MPO assay

The right base lobe of lung was harvested and immediately homogenized on ice in 5 volumes of normal saline. The homogenates were centrifuged at 1 200 r/min for 10 min. The malondialdehyde (MDA) and myeloperoxidase (MPO) content in the supernatants were measured using MDA and MPO assay kit (Nanjing Jincheng Corp., China) following the manufacturer's instructions and expressed as nmol/mg and U/g, respectively.

Lung NF- κ B and ICAM-1 immunohistochemical analysis

Formalin-fixed and paraffin-embedded lung specimens were stained with SP immunohistochemistry technique for NF- κ B and ICAM-1 detection. Experiments were performed following the manufacturer's instructions. Five-micron sections were dewaxed in xylene, cultured in 3% hydrogen peroxide to eliminate intrinsic peroxidase and quenched in normal goat serum for 30 min, then incubated

overnight at 4°C with polyclonal rabbit anti-rat NF- κ B p65 and ICAM-1 antibody (NeoMarkers Corp. and Boster Corp., Ltd., respectively) against purified recombinant NF- κ B or ICAM-1. Then anti-rabbit immunoglobulin and streptavidin conjugated to horseradish peroxidase were added. Finally, 3,3'-diaminobenzidine was used for color development and hematoxylin was used for counter staining. The results were evaluated semi-quantitatively according to the percentage of positive cells in five high power fields at 400 multiple signal magnification.

Western blot analysis of NF- κ B and ICAM-1

Cellular plasma and nuclear protein were extracted from frozen lung tissue with protein extraction kit (Pierce, Meridian Road, Rockford, IL, USA) according to the manufacturer's instructions. Protein concentrations were determined by Coomassie blue dye-binding assay (Nanjing Jincheng Corp, China). Samples were mixed with loading buffer and boiled for 5 min. Thirty micrograms of protein (nuclear protein for NF- κ B p65, plasma protein for ICAM-1) was loaded into each lane of 10% SDS-PAGE gel electrophoresis at 100 V for 4 h. After electrophoresis, the proteins were electroblotted onto NC membranes (Millipore, Bedford, MA, USA) at 9 V for 30 min. Nonspecific binding was blocked by incubation in phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBS-T) and 5% skim milk. The transferred membranes were incubated overnight at 4°C with rabbit polyclonal antibodies NF- κ B p65 and ICAM-1 (NF- κ B p65 at 1:1 000 dilution, ICAM-1 at 1:500 dilution) against rat in PBS-T containing 5% skim milk. After washing thrice in PBS-T, the membranes were incubated with anti-rabbit IgG (Zhongshan Bio., China) conjugated to horseradish peroxidase at a dilution 1:2 000 in PBS-T containing 5% skim milk for 1 h at 37°C. After three additional washes with PBS-T, the signals were visualized by DAB assay kit (Maixin-bio, China) and analyzed with a gel imaging system (Kodak system EDAS120).

Statistical analysis

All data were expressed as mean \pm SD. Statistical analysis was performed using *F*- and *Q*-tests. $P < 0.05$ was considered statistically significant.

RESULTS

Lung pathological and BALF changes

The lung histological structure was normal in control group, while the lung tissues were obviously damaged with edema, hemorrhage, and inflammatory cell infiltration in I/R group. There was a significant difference between I/R and control group in pathological score ($P < 0.01$) and BALF content. After the administration of PDTC, the pathological score of lung injury and BALF content was improved significantly when compared to I/R group ($P < 0.05$, Table 1).

Serum IL-6 level

Compared to control group, serum IL-6 level was significantly increased in I/R group ($P < 0.01$, Table 2).

Table 1 Protein content of lung lavage fluid in different groups (mean±SD)

| Groups | n | Lung lavage fluid (g/L) |
|---------------|---|------------------------------|
| Control group | 8 | 0.8769 ± 0.1622 |
| I/R group | 8 | 1.2462 ± 0.3303 ^b |
| PDTC group | 8 | 0.9299 ± 0.2573 ^a |

^aP<0.05 vs I/R group, ^bP<0.01 vs control.

Table 2 Serum IL-6 level in different groups (mean±SD)

| Groups | n | IL-6 (ng/L) |
|---------------|---|---------------------------|
| Control group | 8 | 22.51 ± 6.10 |
| I/R group | 8 | 42.85 ± 7.35 ^b |
| PDTC group | 8 | 28.08 ± 7.55 ^a |

^aP<0.05 vs I/R group, ^bP<0.01 vs control.

Compared to I/R group, serum IL-6 level was significantly decreased in PDTC treatment group ($P<0.05$).

Lung MDA and MPO assay

Compared to control group, lung MDA and MPO significantly increased in I/R group ($P<0.01$). Compared to I/R group, lung MDA and MPO significantly decreased in PDTC treatment group ($P<0.01$, Table 3).

Immunohistochemical analysis of lung NF-κB and ICAM-1

The expression of NF-κB p65 and ICAM-1 in control group showed light brown immunostaining in cytoplasm and no staining in the nuclei. The significant positive expressions of NF-κB p65 and ICAM-1 as strong brown staining in cytoplasm and nuclei were observed in I/R group ($P<0.01$). Compared to I/R group, the positive rates of NF-κB p65 and ICAM-1 expression decreased significantly in PDTC group ($P<0.01$, Figures 1 and 2).

Western blot analysis of NF-κB and ICAM-1

Western blot showed weak NF-κB p65 and ICAM-1 positive signals in the lungs of control group. In contrast, significant increase of NF-κB p65 and ICAM-1 protein expression was found in I/R group ($P<0.01$). Compared to I/R group, the signals weakened significantly in PDTC group ($P<0.05$, Figure 3 and Table 4).

DISCUSSION

Previous studies have identified many mediators involved in the pathogenesis of lung injury induced by intestinal I/R^[16,17], which exert their effects via a direct toxic action on lung tissue. Recent studies showed that these mediators such as NO, ROS, TNF-α, and ICAM-1 can be regulated by NF-κB. NF-κB is a rapid response transcription factor, which is maintained in the cytoplasm and consists of two subunits of 50 and 65 ku bound to an inhibitor protein, I-κB. This phosphorylated inhibitor unit is tagged by ubiquitin for subsequent proteolysis, and then the free NF-κB complex is able to translocate into the nuclei

Table 3 Lung MDA and MPO level in different groups (mean±SD)

| Groups | n | MDA (nmol/mg) | MPO (U/g) |
|---------------|---|--------------------------|------------------------------|
| Control group | 8 | 1.44 ± 0.17 | 2.6075 ± 0.4372 |
| I/R group | 8 | 2.13 ± 0.39 ^d | 3.8763 ± 0.5682 ^d |
| PDTC group | 8 | 1.50 ± 0.18 ^b | 3.3350 ± 0.4712 ^a |

^aP<0.05; ^bP<0.01 vs I/R group, ^dP<0.01 vs control.

Table 4 IOD level of lung NF-κB p65 and ICAM-1 in different groups (mean±SD)

| Groups | n | IOD level | |
|---------------|---|-----------------------------|----------------------------|
| | | NF-κB p65 | ICAM-1 |
| Control group | 8 | 36.295 ± 4.34 | 31.5 ± 6.87 |
| I/R group | 8 | 124.14 ± 21.22 ^b | 63.55 ± 18.45 ^b |
| PDTC group | 8 | 41.82 ± 9.16 ^a | 45.89 ± 8.86 ^a |

^aP<0.05 vs I/R group, ^bP<0.01 vs control.

where it transactivates target genes^[18,19]. As a consequence, activated polymorphonuclear neutrophils (PMNs) and pro-inflammatory cytokines (TNF-α, ILs) are released into the systemic circulation, interact with the vascular endothelium of distant organs, primarily the lungs, contributing to the systemic inflammatory response^[20]. ICAM-1 is a member of the immunoglobulin superfamily, which is inducible by NF-κB and inflammatory cytokines such as IL-1β and TNF-α. ICAM-1 might be upregulated to involve the adhesion and infiltration of leukocytes into the injured site^[21]. MPO resides in PMNs and its activity reflects the level of accumulation of PMNs.

In our study, 1 h of intestinal ischemia followed by 2 h of reperfusion induced lung injury manifested as a significant increase of BALF content and pathological injury score as well as PMN infiltration. These changes were parallel to the level of lung NF-κB p65, suggesting that the activation of NF-κB is involved in the pathogenesis of lung injury induced by intestinal I/R. As a consequence, activated PMNs and pro-inflammatory cytokines (such as IL-6) are released into the systemic circulation, and interact with the vascular endothelium of organs. Endothelial adhesion molecules expressed on the surface of endothelial cells (such as ICAM-1) play a key role in neutrophil chemoattraction, adhesion and emigration from the vasculature to the tissue, contributing to the systemic inflammatory response and organ injury^[22,23]. The production of ROS, such as lung MDA and MPO (an index of tissue neutrophil count) was observed in I/R group. NF-κB upregulates neutrophil infiltration and expression of ICAM-1. PDTC, as an antioxidant, is involved in its ability to inhibit NF-κB^[24-26] via the stabilization of I-κB-α^[27] or via the inhibition of the ubiquitin-proteasome pathway^[28]. In our study, PDTC reduced the neutrophil infiltration, lung expression of ICAM-1 and MPO activity, which can prevent the development of lung injury.

In conclusion, activation of NF-κB plays an important

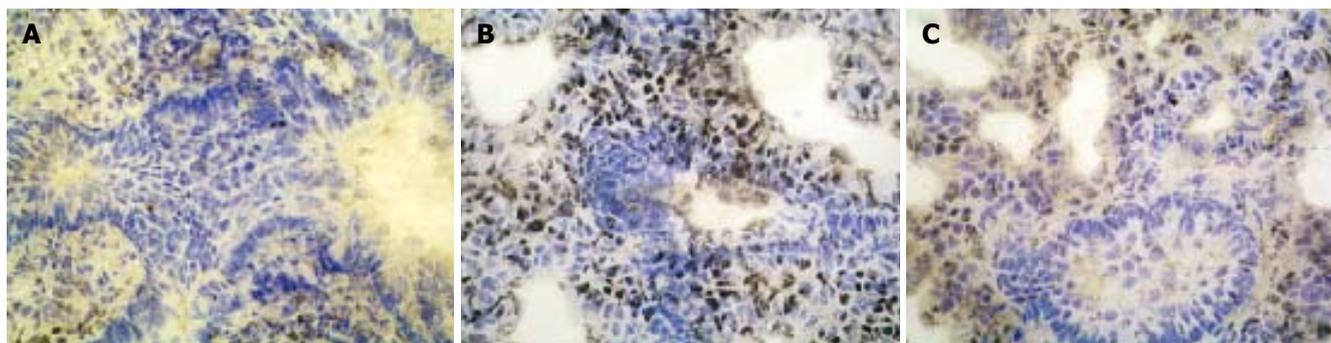


Figure 1 Expression of NF- κ B p65 in lung tissue of control group (A), I/R group (B), and PDTC group (C).

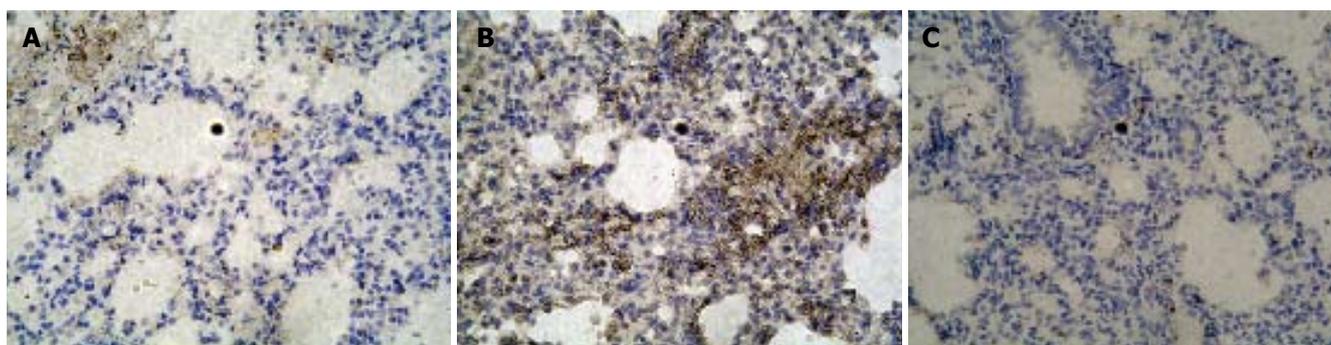


Figure 2 Expression of ICAM-1 in lung tissue of control group (A), I/R group (B), and PDTC group (C).

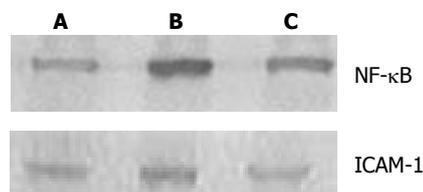


Figure 3 Lung NF- κ B P65 and ICAM-1 protein signals from left to right side in control group (A), I/R group (B) and PDTC group (C). Compared to control group, the signals in I/R group increased significantly and weakened significantly in PDTC group.

role in the pathogenesis of lung injury induced by intestinal I/R by upregulating the neutrophil infiltration and lung ICAM-1 expression. PDTC can prevent lung injury induced by intestinal I/R by inhibiting the activity of NF- κ B.

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Cocultivation of umbilical cord blood CD34⁺ cells with retro-transduced hMSCs leads to effective amplification of long-term culture-initiating cells

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Abstract

AIM: To establish a novel coculture system for ex vivo expansion of umbilical cord blood(UCB) hematopoietic progenitors using thrombopoietin (TPO)/Flt-3 ligand (FL)-transduced human marrow-derived mesenchymal stem cells (tfhMSCs) as feeder.

METHODS: UCB CD34⁺ cells were isolated and cultured using four culture systems in serum-containing or serum-free medium. Suitable aliquots of cultured cells were used to monitor cell production, clonogenic activity, and long-term culture-initiating culture (LTC-IC) output. Finally, the severe-combined immunodeficient (SCID) mouse-repopulating cell (SRC) assay was performed to confirm ability of the cultured cells to reconstitute long-term hematopoiesis.

RESULTS: There were no significant differences in the number of total nucleated cells among different culture systems in serum-containing medium during 21-d culture. However, on d 14, the outputs of CD34⁺ cells, CFU-C and CFU-GEMM in tfhMSCs coculture system were significantly enhanced. LTC-IC assay demonstrated that the tfhMSCs coculture system had the most powerful activity. The severe-combined immunodeficient (SCID) mouse repopulating cell (SRC) assay confirmed extensive ability of the expanded cells to reconstitute long-term hematopoiesis. Furthermore, PCR analysis demonstrated the presence of human hematopoietic cells in the bone marrow and peripheral blood cells of NOD/SCID mice.

CONCLUSION: The TPO/FL-transduced hMSCs, in combination with additive cytokines, can effectively expand hematopoietic progenitors from UCB in vitro and the tfhMSCs coculture system may be a suitable system for ex vivo manipulation of primitive progenitor cells under contact culture conditions.

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Key words: Mesenchymal stem cells; Thrombopoietin; Flt-3 ligand; Hematopoiesis

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INTRODUCTION

Hematopoietic stem cells (HSCs) are generally defined as cells having the self-renewing potential and the capacity to give rise to differentiated cells of all hematopoietic lineages^[1]. Therefore, HSC transplantation is performed for complete healing of hematologic disorders and as a supportive therapy after high-dose chemotherapy against malignant diseases. HSCs can be collected from peripheral blood (PB), bone marrow (BM), and umbilical cord blood (UCB). Human UCB is thought to contain a high number of primitive hematopoietic cells, because the number of severe combined immunodeficiency (SCID)-repopulating cells (SRCs) in nonobese diabetic/SCID (NOD/SCID) mice that had received transplants from UCB was higher than that in NOD/SCID mice that had received transplants from other sources^[2-4]. Moreover, the frequency of graft-versus-host disease, which is a severe side effect of HSC transplantation in patients, is reduced among patients receiving transplants from UCB^[5], and UCB can be obtained from the cord-blood bank network. However, the total number of UCB HSCs harvested from one donor's UCB is limited and is not sufficient for HSC transplantation in an adult patient. To overcome this problem, attention has been increasingly focused on

ex vivo expansion of HSCs. Many approaches have been reported during the last decade, and they can be divided into 2 categories. The first category is treatment of HSCs with various combinations of cytokines. Treatment with the following combinations of cytokines increased the progenitor/stem cell population by 2- to 30-fold in the relatively short period of 10 to 14 d: Flt-3 ligand (FL), stem cell factor (SCF), and thrombopoietin (TPO); SCF, granulocyte-colony stimulating factor (G-CSF), and megakaryocyte growth and development factor (MGDF); FL, SCF, G-CSF, interleukin-3 (IL-3), and interleukin-6 (IL-6); and FL, SCF, and IL-6^[6-9]. However, it is difficult to maintain HSC activity in long-term cultures even if the total number of hematopoietic cells could be expanded. Hence, these methods could be improved for use in clinical settings. The second category involves using stromal cells. It has been reported that the SCID-repopulating activity (SRA) of human HSCs could be maintained by coculture with murine stromal cells for 7 wk^[10], and that the SRA could be maintained by coculture with the AGM-S3 stromal cell line for 4 wk^[11]. MS-5 expanded SRCs for 2 wk^[12]; FBMD-1 expanded cobblestone area-forming cells by 90-fold^[13]; HESS-5 expanded SRCs for only 5 d^[14,15]. Contact between HSCs and stromal cells is important for maintaining the function of HSCs^[16,17]. However, when human HSCs are cocultured with nonhuman stromal cells, the expanded human HSCs might have a risk of being exposed to an unknown viral contamination in animal stromal cells.

Several methods of ex vivo expansion using human primary stromal cells were reported^[18,19]. When HSCs were cocultured with human primary stromal cells, the HSCs were expanded for 2 to 4 wk. However, in general, when human primary somatic cells divide in an in vitro culture, the telomeric DNA at the end of the chromosome shortens at each cell division. Then, the replication of human primary cells slows (aging occurs), and the cells finally cease to divide (crisis phase)^[20,21]. To obtain a sufficient number of primary stromal cells for use on a clinical scale, we have to harvest BM many times, and we cannot ignore the burden on the donor. To solve this problem, trials to establish human stromal cell lines using transduction of viral antigens such as human papillomavirus (HPV) E6/E7 and simian virus 40 (SV40) large T have been reported^[22-24]. These stromal cells could maintain HSCs, but the possibility of transformation was mentioned^[25].

Recently, it was shown that Mesenchymal stem/progenitor cells in human UCB and placenta could support ex vivo expansion of CD34⁺ hematopoietic stem cells^[26,27]. However, no report has investigated whether transduced human marrow-derived mesenchymal stem cells (hMSCs) could be useful in ex vivo expansion of UCB hematopoietic progenitors. Previous research has shown that TPO and FL, the two early-acting cytokines, could lead to significant expansion of HSC populations, including long-term culture-initiating cells (LTC-IC). Our group have introduced TPO and FL genes into cultured hMSCs by retroviral vector transfer and demonstrated long-term expression in vitro and in vivo. In this study, we attempted to establish a coculture system for ex vivo

expansion of UCB hematopoietic progenitors using TPO/FL-transduced hMSCs (tfhMSCs) as feeder. As a result, tfhMSCs is capable of expanding UCB hematopoietic cells in synergy with extra cytokines *in vitro*.

MATERIALS AND METHODS

Analysis of expression of the TPO and FL gene by enzyme-linked immunosorbent assay

Untransduced and TPO/FL-transduced hMSCs (tfhMSCs) were cultured in minimal essential medium α (MEM- α ; HyClone, Logan, UT, USA) supplemented with 100 mL/L fetal bovine serum (FBS; GibcoBRL, GrandIsland, NY, USA) at 37°C with 50 mL/L CO₂ in humidified air. For assaying TPO and FL secretion, untransduced hMSCs and/or tfhMSCs were passaged when cells reached 90% confluence by transferring 2.5×10^6 to 5.0×10^6 cells into a 75-cm² flask with 12 mL of hMSCs medium. Twenty-four hours later, 1 mL of culture supernatant was collected and stored at -80°C. The assay was performed in triplicate using the TPO and FL ELISA kit (BioSource International, Camarillo, CA, USA). The level of TPO and FL was normalized to the level of endogenously expressed IL-6 measured with an IL-6 ELISA kit (BioSource International) using the procedures suggested by manufacturer. Plates were read on a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA) and the data were analyzed using Microsoft Excel.

UCB sample collection and CD34⁺ cell purification

UCB were collected from normal full-term pregnancies according to the regulations of the Research Ethics Committee of Women's Hospital, School of Medicine Zhejiang University. Mononuclear cells (MNC) were isolated using Ficoll-Hypaque (1.077 ± 0.001 Kg/L, Sigma, St. Louis, MO), washed, and resuspended in Iscove's modified Dulbecco's medium (IMDM; HyClone, Logan, UT) supplemented with 100 mL/L fetal bovine serum (FBS; GibcoBRL, GrandIsland, NY). CD34⁺ cell purification utilized positive selection using the *miniMACS* immunomagnetic separation system (Miltenyi Biotec GmbH, Glodbach, Germany) according to the manufacturer's instructions. Briefly, MNCs were suspended in buffer containing phosphate-buffered saline (PBS), 5 mL/L bovine serum albumin (BSA; Sigma), and 2 mmol/L EDTA (BSA-EDTA-PBS), and incubated for 15 min with monoclonal hapten-conjugated anti-CD34 antibody (clone: QBEND/10) and human Ig to prevent nonspecific binding. Washed cells were resuspended in BSA-EDTA-PBS and incubated for 15 min with colloidal super-paramagnetic microbeads conjugated to an anti-hapten antibody. After labeling, the cell suspension was passed through a column (VS⁺ separation column) held within a magnetic field causing CD34⁺ cells to be retained in the column. CD34⁺ cells were collected by removal of the column from the magnet and washing with BSA-EDTA-PBS. Ninety-six percent or more of the enriched cells were CD34⁺ by flow cytometric analysis.

Human cytokines

Recombinant human TPO, granulocyte-macrophage

colony-stimulating factor (GM-CSF), and erythropoietin (EPO) were purchased from Peprotech (London, UK). IL-3 and IL-6 was purchased from RELIATech GmbH (Braunschweig, Germany). Recombinant human SCF was a gift from Amgen Biologicals (Thousand Oaks, CA). Recombinant human FL was purchased from R&D Systems (Minneapolis, MN). The final concentrations of cytokines were as follows: TPO, 50 µg/L; FL, 50 µg/L; IL-3, 20 µg/L; IL-6, 20 µg/L; SCF, 50 µg/L; GM-CSF, 10 µg/L; and EPO, 3 000 U/L.

Culture systems

Stroma-free culture and coculture with tfhMSCs or hMSCs were performed in culture media in 24-well microplates (Costar, Bethesda, MD). Serum-containing liquid culture was carried out using a medium containing 125 mL/L horse serum (HS; HyClone), 125 mL/L FBS, 10⁻⁴ mol/L 2-mercaptoethanol (Sigma), 2 mmol/L L-glutamine (Sigma) and IMDM supplemented with 10⁻⁶ mol/L hydrocortisone (Sigma) with or without feeder layer. In the coculture, tfhMSCs or hMSCs were seeded at 1 × 10⁵ cells per well with MEM-α supplemented with 100 mL/L FBS. After obtaining a confluent feeder layer, cells were washed five times and subjected to γ-irradiation at a dose of 12 Gy. the medium was then changed for coculture. Totally 20 000 UCB CD34⁺ cells were expanded for 21 d under four conditions: 1) tfhMSCs coculture system (tfhMSCs + SCF + IL-3 + IL-6 + GM-CSF); 2) hMSCs coculture system (hMSCs + TPO + FL + SCF + IL-3 + IL-6 + GM-CSF); 3) cytokines culture system (TPO + FL + SCF + IL-3 + IL-6 + GM-CSF); 4) hMSCs (TPO/FL-free) culture system (hMSCs + SCF + IL-3 + IL-6 + GM-CSF). On d 7 and 14 of culture, the medium in each well was removed and replaced with fresh medium. On d 7, 14 and 21 of culture, aliquots of cultured cells were harvested and subjected to cell count, clonal cell culture, and flow cytometric analysis when contamination of stromal cells in the harvested cells was negligible (< 2%) by microscopic visualization. On d 14, cultured cells were harvested and subjected to LTC-IC assay and SRC assay. Short-term (7 d) serum-free liquid culture was carried out using StemProTM-34SFM (GibcoBRL) supplemented with StemProTM-34 Nutrient Supplement (GibcoBRL), 2 mmol/L L-glutamine, and penicillin/streptomycin (GibcoBRL).

Immunophenotyping by flow cytometry

Aliquots of cells were suspended in EDTA-BSA-PBS and incubated with mouse IgG (InterCell Technologies, Hopewell, NJ) to block nonspecific binding. Cells were then reacted for 15 min with FITC- and PE-conjugated monoclonal antibodies at 4°C. Unbound antibodies were removed by two washes, and cells were resuspended in EDTA-BSA-PBS. Stained cells were then passed through a nylon mesh filter and subjected to two-color flow cytometric analysis. Cells labeled with FITC- and PE-conjugated mouse isotype-matched antibodies were used as negative controls. The analysis was performed using an FACSort flow cytometer (Becton Dickinson, San Jose, CA) with CELLQUESTTM software (Becton Dickinson). At least 10 000 events were acquired for each analysis.

Antibodies used were as follows: FITC-conjugated CD14, CD15, CD19, CD33, CD34, and CD41; PE-conjugated CD38 and CD45 antibodies. Glycophorin A antibodies were from Immunotech (Marseille, France). CD14, CD33, and CD45 antibodies were from Pharmingen (San Diego, CA) and all others were from Becton Dickinson. Furthermore, in some experiments, aliquots of cultured cells were subjected to three-color flow cytometric analysis to assess the lineage commitment of progenitors. Samples were incubated for 15 min with biotin-conjugated anti-CD34 (Immunotech, Marseille, France). Cells labelled with a biotin-conjugated mouse isotype-matched antibody were used as a negative control. After washing, cells were labeled with streptavidin PerCP (Becton Dickinson), PE-conjugated anti-CD38, and various FITC-conjugated monoclonal antibodies. Three-color flow cytometry was performed using an FACSCalibur (Becton Dickinson) with CellQuest software (Becton Dickinson).

Colony-forming cell assay (CFC assay)

Aliquots from initial UCB samples or cultured cells were incubated in methylcellulose media at concentrations of 1-2 × 10⁵ cells/L for purified CD34⁺ cells and 5-10 × 10⁵ cells/L for cultured cells in 35-mm tissue culture dishes (Costar). One milliliter of culture mixture contained 12 mL/L 1500 cp methylcellulose (Sigma), MEM-α, 10 g/L deionized fraction V BSA (Sigma), 10⁻⁴ mol/L 2-mercaptoethanol, 300 mL/L fetal calf serum (JRH Biosciences, Lenexa, KS), EPO, IL-3, SCF, GM-CSF, and cells. Dishes were incubated at 37°C in a humidified atmosphere with 50 mL/L CO₂ in air. All cultures were done in triplicate. Total colony-forming units in culture (CFU-C) and mixed colonies containing erythroid and myeloid cells and megakaryocytes (CFU-GEMM) consisting of 50 or more cells were scored under an inverted microscope at 21 d of culture. To assess the accuracy of in situ identification, individual colonies were lifted with an Eppendorf micropipette under direct microscopic visualization, spread on glass slides using a cytocentrifuge and studied with May-Grunwald-Giemsa staining.

Long-term culture-initiating cell assay (LTC-IC assay)

LTC-IC assay was performed as described by Sutherland *et al.*^[28], with slight modifications. Briefly, bone marrow stromal cells derived from hematologically normal donors were seeded at 10⁵ cells per well in 96-well flat-bottomed plates (Costar) with MEM-α supplemented with 100 mL/L FBS. After obtaining semiconfluent feeder layers, stromal cells were irradiated with 15-Gy using a ⁶⁰Co γ-irradiator. CD34⁺ cell subpopulations purified from UCB or those isolated from cultured cells by sorting with an FACSVantage (Becton Dickinson) were seeded at limiting dilution on the feeder layer in serum-containing media. For each evaluation, at least three cell concentrations were used with 24 replicates per concentration. Culture plates were incubated at 37°C with 50 mL/L CO₂ in air and weekly changes of medium. After 5 wk of culture, cells were assayed for CFU-C in methylcellulose medium. Colonies were scored 2 wk later. The frequency of wells in which there were no clonogenic progenitors was determined

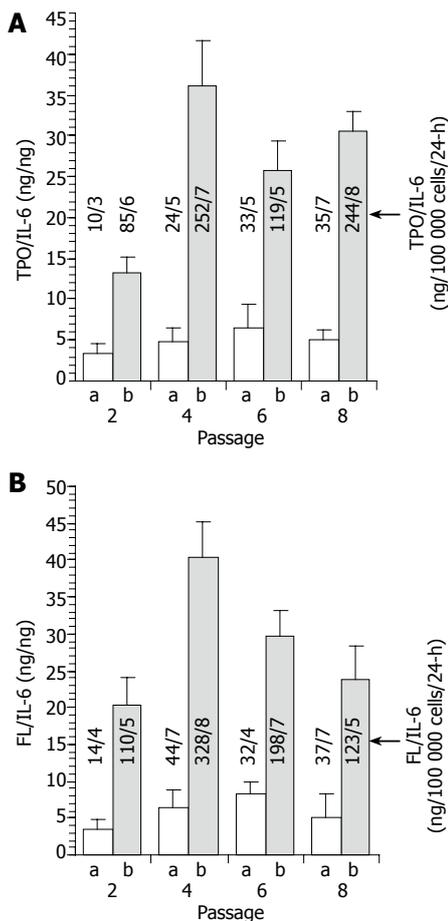


Figure 1 Maintenance of TPO and FL production *in vitro*. TPO (A) and FL (B) levels secreted from tfhMSCs and untransduced hMSCs and endogenous IL-6 levels were assayed in the medium after 2, 4, 6, and 8 passages in culture. The level of TPO and FL in the supernatant was normalized to the level of endogenous IL-6 and the ratio was plotted for passage number. In addition, the absolute values (ng/100 000 cells/24 h) of TPO, FL and IL-6 are shown (arrow). Data were obtained from triplicate ELISA measurements. a=untransduced hMSCs; b=tfhMSCs.

according to the number of the initial input population. Poisson statistics were applied to the single-hit model and the frequency of LTC-IC was calculated with the maximum likelihood estimator.

SCID-repopulating cells assay (SRC assay)

SRC assay was performed as previously described^[29], with slight modifications. Briefly, 8-wk-old male NOD/Shi-scid (NOD/SCID) mice were obtained from the Central Institute for Experimental Animals, Shanghai Institutes for Biological Sciences, CAS. All animals were handled under sterile conditions and maintained under microisolators in the animal facility located at Zhejiang Academy of Medical Sciences. Human hematopoietic cells at the indicated doses were transplanted by tail-vein injection into sublethally irradiated mice (350 cGy using a linear accelerator). Cells were co-transplanted with irradiated (15 Gy using a ⁶⁰Co γ -irradiator) nonrepopulating CD34⁺ cells as accessory cells. Mice were killed 7 wk after transplantation, and the bone marrow (from the femurs and tibiae) and peripheral blood cells (from the retro-orbital venous plexus using heparin-coated micropipettes) were harvested. The

presence of human hematopoietic cells was determined by detection of cells positively stained with FITC-conjugated antihuman CD45 using flow cytometry. Polymerase chain reaction (PCR) analysis using human Alu sequence primers (5' - GTGGGCGACAGAACGAGATTCTAT; 5' - CTCCTACTTGGAGACAGGTTCA) was also performed to confirm flow cytometric results.

Statistical analysis

Results are expressed as mean \pm SD. Statistical comparisons were performed using the two-sided Student's *t*-test. Iterative approximation of Newton's method was performed using Microsoft Visual Basic 6.0 software.

RESULTS

Analysis of expression of TPO and FL gene from tfhMSCs and untransduced hMSCs

We transduced hMSCs with the secreted cytokine TPO and FL, which allows direct quantitation of the extracellular product. Human MSCs were transduced with a retroviral vector (pLXINTF) expressing TPO, FL and neomycin followed by selection in the presence of G418 for 2 wk. Cultures were maintained and expanded further for up to 8 passages. Twenty-four hours after each replating aliquots of supernatant from tfhMSCs and untransduced hMSCs was removed for measurement of secreted TPO and FL. The TPO and FL secretion value obtained was normalized to the secretion value obtained for endogenous human IL-6 measured in the same sample aliquot. The absolute values for each cytokine are shown in Figure 1. We chose IL-6 as the control cytokine because expression was in a range similar to that of the transduced gene product and previous data showed similar expression of IL-6 from hMSCs over time in hMSC cultures^[30].

We observed TPO transgene expression from tfhMSCs and untransduced hMSCs averaging 175 ± 85 and 25 ± 11 ng/ 10^5 cells/24 h respectively (Figure 1A) and FL transgene expression averaging 190 ± 99 and 31 ± 12 ng/ 10^5 cells/24 h respectively (Figure 1B); Endogenous IL-6 protein levels averaging 6 ± 2 ng/ 10^5 cells/24 h. The cytokine ratio (transduced/endogenous) of tfhMSCs demonstrated a 14- to 36-fold and 22- to 41-fold increase respectively in TPO and FL secretion over endogenous IL-6 expression. The results demonstrate that *in vitro* transgene expression from tfhMSCs was maintained for at least three months in culture.

Assessment of the supportive effects of tfhMSCs on proliferation of UCB primitive progenitor cells (PPC) in synergy with extra cytokines

To determine whether tfhMSCs was capable of supporting *ex vivo* expansion of UCB-derived hematopoietic cells, four culture systems were established as shown in materials and methods. In the stroma-containing culture, tfhMSCs or untransduced hMSCs were irradiated and cocultured with CD34⁺ cells from UCB, and in the stroma-free culture only cytokines were used for expansion. The total number of nucleated cells, CD34⁺ cells, CFU-C

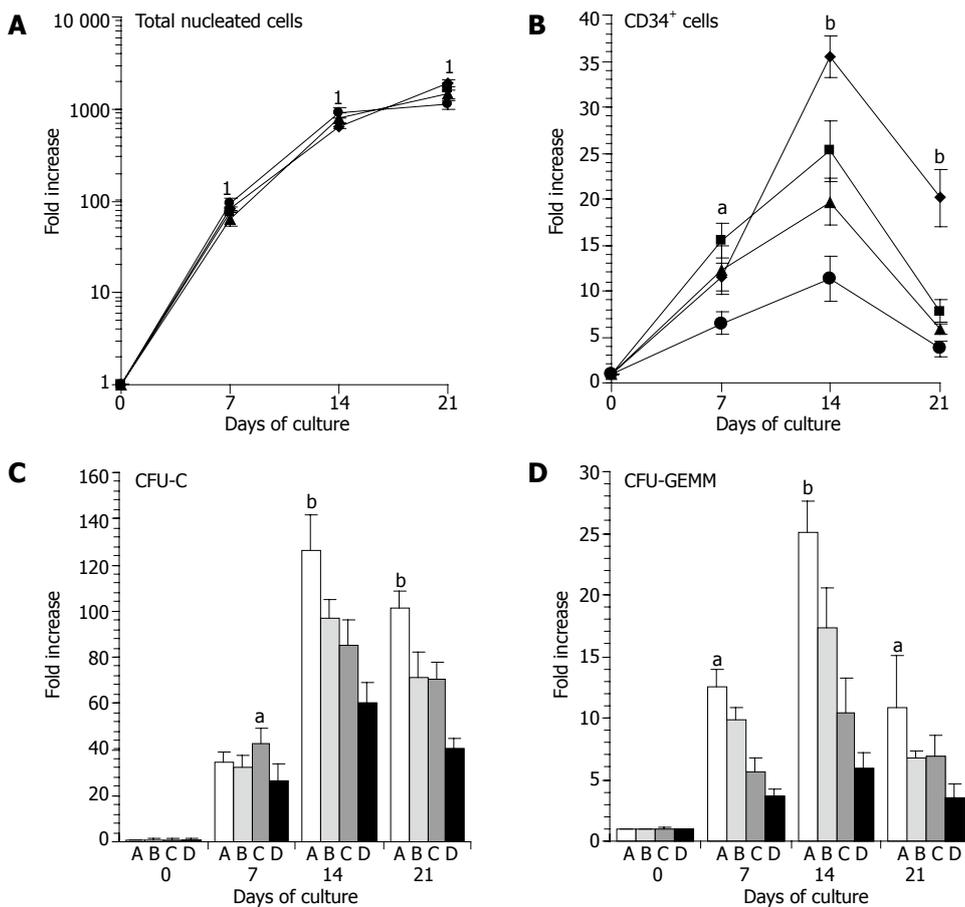


Figure 2 Effects of several culture systems on ex vivo expansion of hematopoietic progenitors. UCB CD34⁺ cells were cultured in serum-containing medium using four culture systems. **A:** Total nucleated cells; **B:** CD34⁺ cells; **C:** Total colony-forming units in culture (CFU-C); **D:** Mixed colonies containing erythroid cells and megakaryocytes (CFU-GEMM). The results represent the mean fold increase \pm SD of three different experiments on d 7, 14, and 21 of culture. ¹No significant differences between different culture systems. ^a $P < 0.05$, ^b $P < 0.01$ as compared with other three groups. \blacklozenge = tfhMSCs coculture system; \blacksquare = hMSCs coculture system; \blacktriangle = cytokines culture system; \bullet = hMSCs (TPO/FL-free) culture system. A = tfhMSCs coculture system; B = hMSCs coculture system; C = cytokines culture system; D = hMSCs (TPO/FL-free) culture system.

and CFU-GEMM was evaluated on d 7, 14 and 21. CD34 and CD38 expression among hematopoietic cells in four culture conditions were also determined on d 14.

We first assessed the effect of tfhMSCs on ex vivo expansion of UCB-PPC in the serum-containing culture. As a result, tfhMSCs alone could not effectively support proliferation of UCB hematopoietic progenitors (data not shown). The effects of four culture systems on ex vivo expansion of total nucleated cells and CD34⁺ cells were then studied in serum-containing medium. During culture, there were no significant differences in the number of total nucleated cells among different culture systems (Figure 2A). The expansion magnitude of CD34⁺ cells by tfhMSCs coculture system (11.52 ± 1.51 fold) and cytokines culture system (12.32 ± 2.69 fold) was lower than that by hMSCs coculture system (15.40 ± 1.89 fold) on d 7 ($P < 0.05$), and hMSCs (TPO/FL-free) culture system (6.59 ± 1.26 fold) manifested the lowest expansion capacity ($P < 0.01$) among the four groups (Figure 2B). However, on d 14, CD34⁺ cells were generated more by the tfhMSCs coculture system (35.42 ± 2.25 fold) than by hMSCs coculture system (25.24 ± 3.32 fold), cytokines culture system (19.75 ± 2.56 fold) and hMSCs (TPO/FL-free) culture

system (11.32 ± 2.48 fold) ($P < 0.01$) (Figure 2B). Although fold increase of CD34⁺ cells was largely decreased among all the four systems on d 21, the expansion magnitude of CD34⁺ cells by tfhMSCs coculture system (20.15 ± 3.16 fold, $P < 0.01$) was higher than that by the three systems (Figure 2B).

Next, we determined the outputs of CFU-C and CFU-GEMM. The expansion magnitude of CFU-C in tfhMSCs coculture system (126.54 ± 15.42 fold), although lower than cytokines culture system on d 7 ($P < 0.05$), was much higher than other three culture systems on d 14 ($P < 0.01$). Interestingly, tfhMSCs coculture system significantly stimulated production of CFU-C on d 21 (101.12 ± 7.23 fold, $P < 0.01$) (Figure 2C). The output of CFU-GEMM in tfhMSCs coculture system on d 7, 14 and 21 was also enhanced (12.65 ± 1.36 fold, 25.16 ± 2.53 fold and 10.93 ± 4.12 fold, respectively) ($P < 0.01$) (Figure 2D). Analysis for expression of CD34 and CD38 by flow cytometry among the four culture systems on d 14 was shown in Figure 3.

Ex vivo expansion of UCB CD34⁺ cells in a short-term (7 d) serum-free tfhMSCs coculture system

The synergistic effects of tfhMSCs and additive cytokines

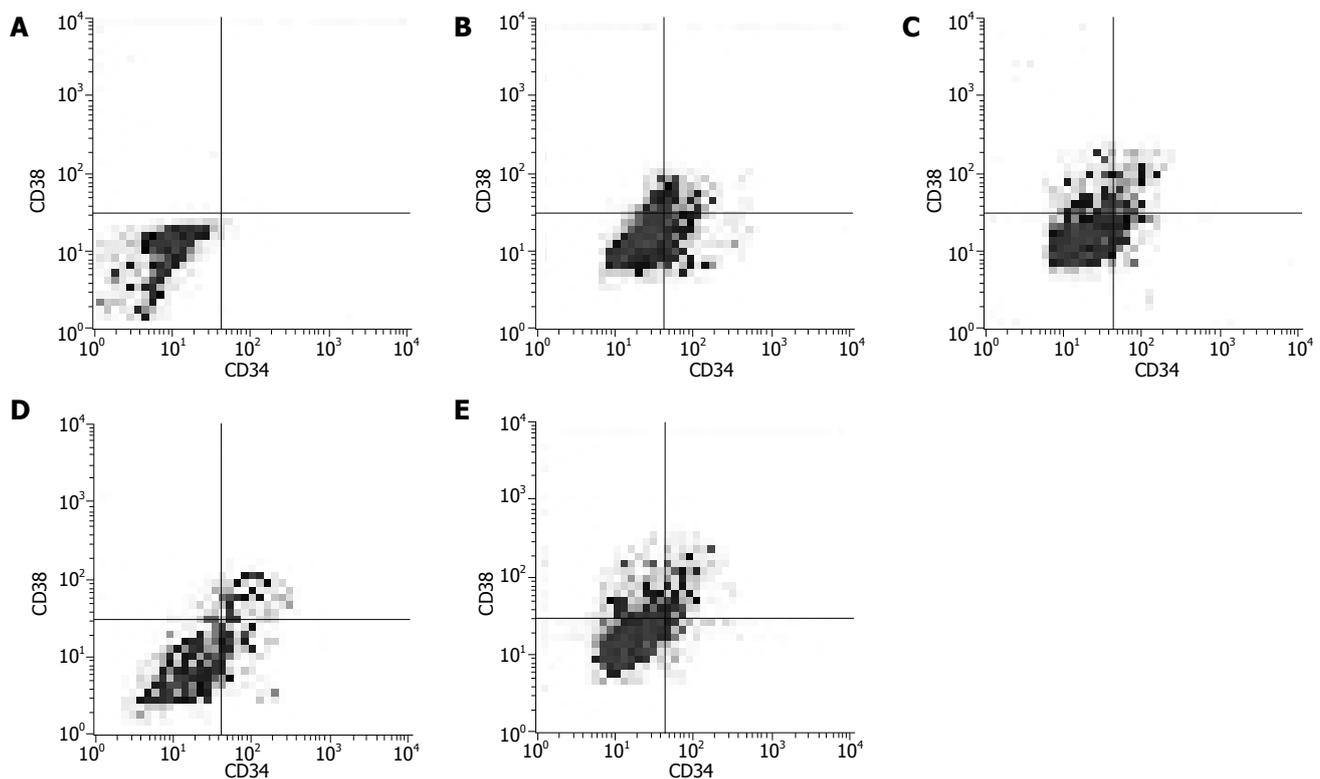


Figure 3 Comparison of CD34 and CD38 expression among hematopoietic cells in different culture systems. CD34⁺ cells derived from a single delivery were cultured using different culture systems in serum-containing medium. On d 14 of culture, aliquots of harvested cells were subjected to flow cytometric analysis. **A**=Negative control; **B**=tfhMSCs coculture system; **C**=hMSCs coculture system; **D**=cytokines culture system; **E**=hMSCs (TPO/FL-free) culture system.

on ex vivo expansion of UCB hematopoietic progenitors was also studied in serum-free culture for a short duration. After 7 d of culture, the number of total nucleated cells in tfhMSCs coculture system was remarkably increased ($P < 0.01$) (Table 1). As a result, the mean number of total nucleated cells was approximately 50 to 90 times the initial input number. CD34⁺ cells were generated more by tfhMSCs coculture system than by other three groups ($P < 0.01$); the number of CD34⁺ cells was over 12 times the initial input number. The outputs of CFU-C and CFU-GEMM in tfhMSCs coculture system were also increased approximately 40-fold ($P < 0.05$) and 15-fold ($P < 0.01$), respectively.

LTC-IC assay using the CD34⁺ population isolated from cells cultured in four culture systems

To determine whether cells generated in four culture systems could preserve the ability to sustain long-term hematopoiesis, the LTC-IC frequency in cells cultured by these systems was quantified. Initially, isolated UCB CD34⁺ cells were cultured for 14 d using four culture systems in the serum-containing culture. Cultured cells were harvested and subjected to a second CD34⁺ cell purification by sorting. LTC-IC assay was performed using sorted CD34⁺ cell populations, as well as those initially prepared from UCB (control samples). The LTC-IC frequency was determined as previously described^[28]. As shown in Table 2, Although the yields of LTC-IC expansion in hMSCs coculture system (5.32 ± 1.73 fold) was higher than cytokines culture system (3.58 ± 1.48 fold) and hMSCs (TPO/FL-free) culture system (2.79 ± 0.56

fold), the tfhMSCs coculture system demonstrated the most powerful activity (10.23 ± 2.89 fold, $P < 0.01$). The findings suggest that the tfhMSCs coculture system might be a novel as well as efficient culture system for UCB-derived hematopoietic progenitor cells.

Effects of coculture system using tfhMSCs as feeder layer on human reconstituting hematopoietic progenitors

Accordingly, we studied the SRC assay to determine whether cells cultured in the tfhMSCs coculture system were capable of long-term multilineage reconstitution *in vivo*. Purified 1×10^5 UCB CD34⁺ cells were initially cultured for 14 d using the tfhMSCs coculture system; harvested cells were then transplanted into NOD/SCID mice. As controls, uncultured 100 000 UCB CD34⁺ cells obtained from the same sources were also transplanted into other mice. Seven weeks after transplantation, human CD45⁺ cells were found in the bone marrow, and peripheral blood cells of mice transplanted with the cultured cells (Figure 4A, and data not shown). There were marked differences in the percentage of chimerism between bone marrow cells in mice transplanted with cultured cells and those transplanted with control samples. Human CD45⁺ cells in the murine bone marrow were further subjected to flow cytometric analysis to determine multilineage reconstitution. As a result, human CD45⁺ cells were positive for CD34, CD33, CD14, CD41, glycophorin A, or CD19 (Figure 4B). Furthermore, PCR analysis demonstrated the presence of human hematopoietic cells in the bone marrow and peripheral blood cells of NOD/SCID mice (Figure 5).

Table 1 Evaluation of effects of four culture systems on ex vivo expansion of hematopoietic progenitors in short-term serum-free culture

| Culture systems | Total nucleated cells | CD34 ⁺ cells | CFU-C | CFU-GEMM |
|------------------------------------|-------------------------|-------------------------|-------------------------|-------------------------|
| tfhMSCs coculture system | 87.56±9.51 ^b | 12.39±2.34 ^b | 40.59±6.23 ^a | 15.84±3.96 ^b |
| hMSCs coculture system | 53.23±6.79 ^a | 9.87±2.89 ^a | 38.78±4.28 ^a | 10.57±2.58 ^b |
| Cytokines culture system | 46.25±4.89 | 9.58±1.26 ^a | 27.68±3.32 | 7.19±1.96 |
| hMSCs (TPO/FL-free) culture system | 48.52±6.49 | 6.79±0.62 | 32.85±4.35 | 5.67±1.09 |

^a*P*<0.05, ^b*P*<0.01 *vs* as compared with hMSCs (TPO/FL-free) culture system.

Table 2 Results of LTC-IC assay using UCB CD34⁺ cells or those generated by four culture systems

| Culture systems | LTC-IC frequency | | Fold LTC-IC amplification |
|------------------------------------|------------------|-----------------------|---------------------------|
| | Pre-expansion | Post-expansion | |
| hMSCs (TPO/FL-free) culture system | 1/619.57±120.89 | 1/102 258.56±9 568.49 | 2.79±0.56 |
| Cytokines culture system | 1/625.38±154.26 | 1/84 112.78±10 867.64 | 3.58±1.48 |
| hMSCs coculture system | 1/621.76±138.59 | 1/93 773.42±20 346.23 | 5.32±1.73 |
| tfhMSCs coculture system | 1/617.43±119.76 | 1/24 140.96±7 586.62 | 10.23±2.89 ^b |

^b*P*<0.01 *vs* calculated by the Student's *t*-test, when data of the tfhMSCs coculture system were compared with data in the other groups.

DISCUSSION

In this study we demonstrated that TPO/FL-transduced human marrow-derived mesenchymal stem cells (tfhMSCs) could effectively support ex vivo expansion of UCB-PPC in synergy with human cytokines. We assessed the supportive effects of four culture systems on proliferation of PPC in serum-containing liquid medium. Although there were no significant differences in the number of total nucleated cells among the different culture systems during culture, tfhMSCs coculture system could dramatically enhance generation of CFU-C, CFU-GEMM and CD34⁺ cells, and more importantly was capable of expanding LTC-IC.

In ex vivo expansion of human UCB-PPC, there are several benefits to using the tfhMSCs as the feeder: (1) tfhMSCs can be maintained easily; (2) consistent hematopoietic-supportive effects are repeatedly obtained; (3) additive TPO and FL are not needed in culture system. The effects of tfhMSCs coculture system were comparable to those obtained by classical culture, including cytokines culture and contact culture. Although contamination of tfhMSCs into cultured cells is a problem, and it is difficult to harvest cultured hematopoietic cells completely, since a number of cultured cells migrate under feeder layers, the tfhMSCs coculture system was considered to be a suitable system for ex vivo manipulation of PPC under stroma-contact culture conditions. Moreover, the tfhMSCs, unlike the primary stromal cells^[17,18], could be expanded and cryopreserved without transformation. Thus, we can prepare a large quantity of these human tfhMSCs at any time. Taking advantage of this culture system, clinical research on ex vivo expansion could be facilitated. For example, progenitor cells such as CD34⁺ cells and CFU-C were extensively expanded more than 125-fold in this system in 2 wk, and it may be possible to use these

expanded cells as a new source of blood transfusion after differentiation of the expanded cells into megakaryocytes or erythroblast progenitor cells^[31].

Although the present study and others^[5,8,32] have demonstrated that addition of TPO and FL in culture systems could lead to significant expansion of HSC populations, including LTC-IC, the tfhMSCs coculture system, which have no addition of TPO and FL, manifested higher expansion of LTC-IC. The mechanism of supportive effect of tfhMSCs remains unknown. Previous investigations have shown that the very hematopoietic cell types whose maximum proliferation in vitro depends on stimulation by the highest concentrations of cytokines^[32]. The ability that tfhMSCs expressing TPO and FL can maintain a higher concentrations of TPO and FL in medium may be a reason.

Serum-free culture could prevent UCB primitive-cell differentiation^[14], and when a serum-free medium is used, the risks of contamination of heterogenic antigen and infectious danger were small^[33]. We then assessed the supportive effects of four culture systems on proliferation of PPC in serum-free liquid medium. We attempted ex vivo manipulation for a short duration. As a result, adequate expansion of UCB-PPC was obtained in tfhMSCs coculture system. Furthermore, compared with serum-containing culture the number of CD34⁺ cells was not increased and the total nucleated cells were not significantly decreased during culture; However, the output of CFU-C and CFU-GEMM was enhanced, suggesting that serum-free conditions prevent primitive-cell differentiation.

Previous findings showed that UCB LTC-IC were present among the CD34⁺ cell fraction^[34,35]. Furthermore, Bhatia *et al.* identified the SRC that were capable of multilineage reconstitution of human hematopoiesis in the bone marrow of NOD/SCID mice^[29]. Therefore, the

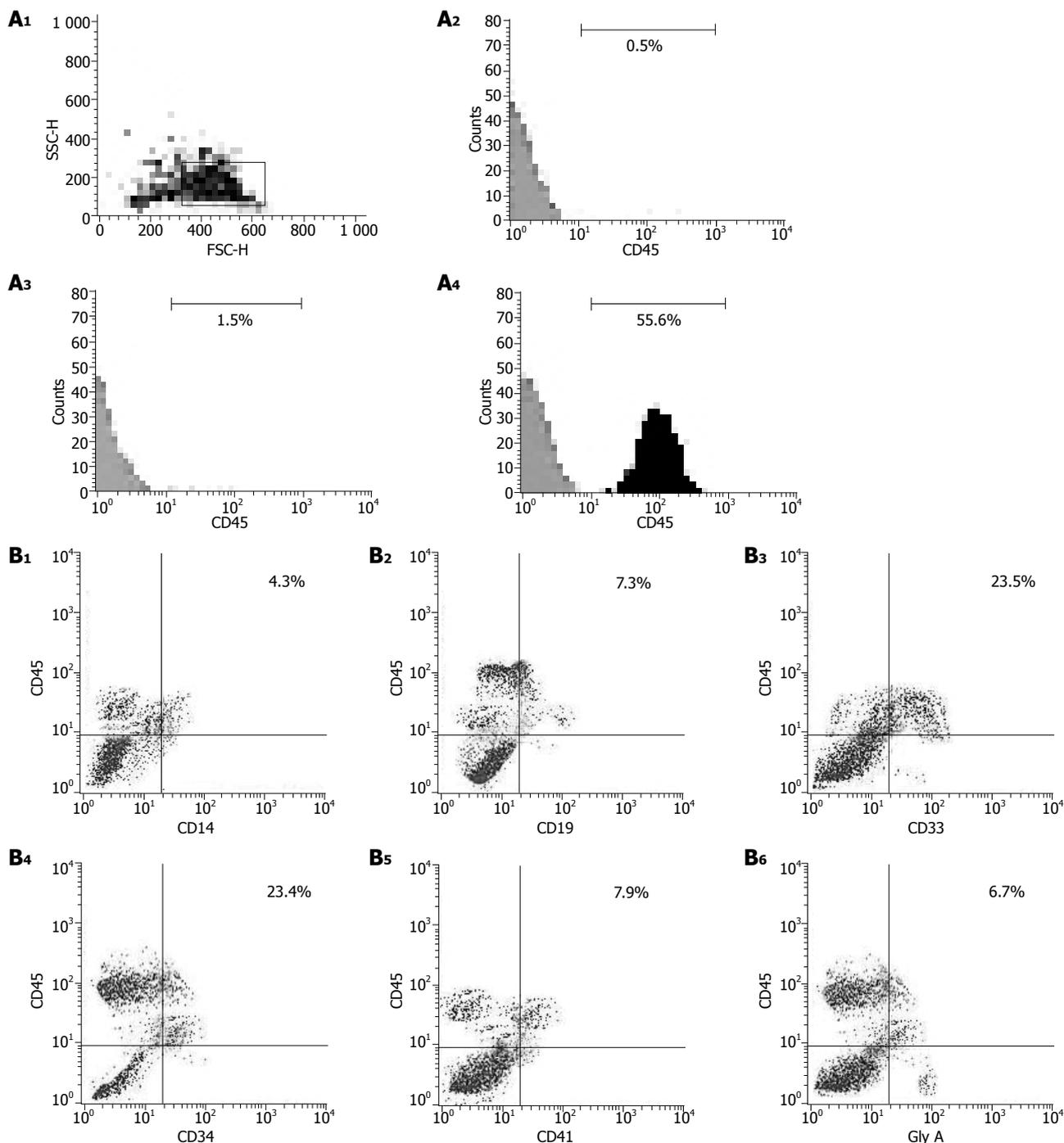


Figure 4 Determination of human hematopoietic reconstitution in NOD/SCID mice 7 wk after transplantation. **A:** Expression of human CD45 on bone marrow cells (BMC) collected from NOD/SCID mice 7 wk after transplantation; (**A₁**): Cells in the gated area were analyzed by flow cytometry; (**A₂**): Bone marrow cells (BMC) from an untransplanted mouse (negative control); (**A₃**): BMC from a mouse transplanted with 1×10^5 human CD34⁺ cells before culture; (**A₄**): BMC from a mouse transplanted with cells obtained after 14-d cultivation of those 100 000 human CD34⁺ cells in the tfhMSCs coculture system; **B₁-B₆**: Specific subsets of human CD45⁺ cells in bone marrow cells of the NOD/SCID mouse transplanted with UCB cells after the coculture. Harvested cells were stained with PE-conjugated CD45 and various FITC-conjugated monoclonal antibodies.

expanded hematopoietic progenitors were expected to sustain long-term hematopoiesis. As a result, 10.23-fold LTC-IC amplification was observed in cells expanded by the tfhMSCs coculture system, although LTC-IC frequency was decreased during culture. The SRC assay indicated the reconstituting ability of these cultured human PPC. Although we could not perform a quantitative SRC assay, the difference in the percentage of chimerism of human CD45⁺ cells between bone marrow cells of mice

transplanted with cultured cells and those transplanted with control samples strongly suggested the extensive ability of these ex vivo-generated PPC to sustain and reconstitute long-term human hematopoiesis in vivo. Furthermore, PCR analysis confirmed flow cytometric results.

In conclusion, the main obstacle to UCB transplantation in adult recipients is the insufficiency of hematopoietic progenitors. A novel tfhMSCs coculture

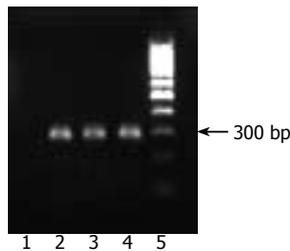


Figure 5 PCR analysis of human Alu sequence in NOD/SCID mice 7 wk after transplantation. DNA was extracted from the bone marrow and peripheral blood cells of NOD/SCID mice 7 wk after transplantation. PCR analysis demonstrated the presence of human hematopoietic cells in the bone marrow (lane 2) and peripheral blood cells (lane 3) of NOD/SCID mice. DNA extracted from human UCB cells was used as a positive control (lane 4). Negative control is shown in lane 1 and DNA marker is shown in lane 5.

system that could efficiently expand UCB hematopoietic progenitors is successfully established. The extent of LTC-IC expansion shown herein has important practical implications in terms of clinical hematopoietic stem cell transplantation.

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Role of Kupffer cells in acute hemorrhagic necrotizing pancreatitis-associated lung injury of rats

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significantly ameliorated by pretreatment with GdCl₃ and KCs play a vital role in AHNP-LI.

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Key words: Pancreatitis-associated lung injury; Kupffer cell; NF-κB; Gadolinium chloride

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Abstract

AIM: To investigate the role of Kupffer cells (KCs) in acute hemorrhagic necrotizing pancreatitis-associated lung injury (AHNP-LI).

METHODS: Forty-two rats were allocated to four groups [sham operation, AHNP model, gadolinium chloride (GdCl₃) pretreatment, GdCl₃ control]. In GdCl₃ pretreatment group, GdCl₃ was administered by caudal vein injection 24 h before the AHNP model induction. Blood from the iliac artery, alveolar macrophages and tissues from the pancreas and lung, were collected in six animals per group 3 and 6 h after acute pancreatitis induction. TNF-α, IL-1 of serum, myeloperoxidase (MPO) of lung tissue, NF-κB activation of alveolar macrophages were detected. Serum AST and ALT in sham operation group and GdCl₃ control group were tested. In addition, histopathological changes of the pancreas and lung were observed under light microscope.

RESULTS: MPO of lung tissue and TNF-α, IL-1 levels of serum were all reduced significantly in GdCl₃ pretreatment group compared to those in AHNP group ($P < 0.01$). NF-κB activation of alveolar macrophages was also attenuated significantly in GdCl₃ pretreatment group compared to that in AHNP group ($P < 0.01$). The pathological injury of the lung was ameliorated obviously in GdCl₃ pretreatment group compared to that in AHNP group. Nevertheless, the serum amylase level did not reduce and injury of the pancreas was not prevented in GdCl₃ pretreatment group.

CONCLUSION: Pulmonary injury induced by AHNP is mediated by KC activation and AHNP-LI can be

INTRODUCTION

Acute lung injury is a severe complication of acute hemorrhagic necrotizing pancreatitis (AHNP)^[1-3]. However, its pathogenic mechanism is not well understood and its treatment remains supportive. Recent researches suggest that inflammatory cytokines derived from the liver, especially hepatic cytokine released from Kupffer cells (KCs) may cause distant organ failure and death in severe pancreatitis and that KCs are an important source of inflammatory cytokines and may be the main factor causing lung damage in AHNP^[4,5]. Here we studied the KC contribution to lung injury associated with AHNP. The present study was to investigate the role of KCs in acute hemorrhagic necrotizing pancreatitis-associated lung injury.

MATERIALS AND METHODS

Experimental animals, agents, and ELISA kits

Male Wistar rats were provided by Experimental Animal Center of Capital Medical University (Beijing). Sodium taurocholate and gadolinium chloride (GdCl₃) were purchased from Sigma-Aldrich (St. Louis, MO, USA). TNF-α, IL-1 ELISA kits were purchased from TPI Ltd. (USA). TransAMTM NF-κB P⁶⁵ chemi ELISA kit was provided by Active Motif (USA). Central LB 960 microplate luminometer was from Berthold Ltd.

Induction of AHNP model

Experimental AHNP was induced as previously described^[6]. Briefly, a small median laparotomy was performed, then the pancreas was exteriorized and the hepatic duct was closed at the liver hilum with a soft microvascular clamp to prevent reflux of the infused

material into the liver. The biliopancreatic duct was cannulated through the duodenum and 5% sodium taurocholate (1 mL/kg body weight, 0.1 mL/min) was retrogradely injected into the biliopancreatic duct. The clamp was removed 5 min after the injection. The abdominal wound was closed, and the animals were sent back to their cages with free access to water and food after surgery.

Animals

Forty-two male Wistar rats (weighing 250-280 g) were randomly divided into four groups: sham operation, AHNP model, GdCl₃ pretreatment and GdCl₃ control. In the GdCl₃ pretreatment group, GdCl₃ solution (4%, 10 mg/kg) was administrated by caudal vein injection 24 h before the AHNP model was established. Blood from the iliac artery, alveolar macrophages, and tissues from the pancreas and lung, were collected in six animals per group 3 and 6 h after acute pancreatitis induction. Serum levels of TNF- α and IL-1 were determined by enzyme-linked immunosorbent assay. Myeloperoxidase (MPO) level in the lung and NF- κ B activation of the alveolar macrophages were detected. Serum AST and ALT in sham operation group and GdCl₃ control group were tested by biochemical method. In addition, histopathological changes of the pancreas and lung were observed under light microscope.

Alveolar macrophage isolation and nuclear protein extraction

Bronchoalveolar lavage (BAL) was performed five times in the left lung, using 5 mL of sterile normal saline per lavage given through a tracheal cannula^[7]. The whole BAL fluid (BALF) was centrifuged at 280 r/min for 10 min at 4 °C. Cell pellets were resuspended (1×10⁵ cells/mL) in RPMI 1640 medium. Cell suspension was then placed in plastic petri dishes (Nunc, Denmark) and incubated at 37 °C for 1 h in a CO₂ incubator (50 mL/L CO₂+95% air). Non-adherent cells were removed from adherent macrophages by washing with RPMI 1640 medium. Purified alveolar macrophages were recovered by gently rubbing the dishes with a rubber policeman. Nuclear protein was extracted from purified alveolar macrophages as previously described^[8]. Protein content was determined by Bradford method, stored at -70 °C for subsequent examination of NF- κ B activity.

Amylase estimation

Serum amylase activity was measured by iodoamylum method and expressed as U/L.

Serum TNF- α , IL-1 estimation

Serum TNF- α and IL-1 were measured by ELISA method according to the instructions of the kits.

Lung tissue MPO estimation

Lung tissue MPO activity was detected according to the instructions of commercial kit.

Serum AST and ALT estimation

Serum levels of AST and ALT were determined using Toshiba VF-A5/A5P Bio-Chemical analyzer.

Table 1 Serum amylase in all experimental groups (U/L, mean±SD)

| Group | Serum amylase | |
|--------------------------------|-------------------------------|-------------------------------|
| | 3 h (n=6) | 6 h (n=6) |
| Sham | 499.4±86.3 | 503.8±91.2 |
| AHNP | 10 444.5±1 863.2 ^b | 13 316.4±1 374.1 ^b |
| GdCl ₃ pretreatment | 9 107.1±569.9 ^b | 12 420.4±1 779.2 ^b |

^bP<0.001 vs sham group.

Table 2 Influence of GdCl₃ on hepatic functions of experimental animals (U/L, mean±SD)

| Group | n | Serum AST | Serum ALT |
|---------------------------|---|-----------|-----------|
| Sham 3 h | 6 | 74.2±13.6 | 31.6±9.3 |
| Sham 6 h | 6 | 71.3±15.0 | 32.7±11.3 |
| GdCl ₃ control | 6 | 82.5±14.6 | 28.9±8.7 |

NF- κ B of alveolar macrophage estimation

NF- κ B activation of alveolar macrophages was determined using TransAMTM NF- κ B P⁶⁵ chemi ELISA kit by Central LB 960 microplate luminometer and expressed as relative light units (RLU).

Histology

Pancreas and lung tissues were collected and evaluated by a pathologist blinded to the experimental assignment of the animals. After embedded with paraffin, the tissue was stained with hematoxylin-eosin (H&E). Pulmonary lesion was scored following Lei's criteria^[9].

Statistical analysis

All data were expressed as mean±SD. Comparisons among multiple experimental groups and between each time point were made using ANOVA. P<0.05 was considered statistically significant.

RESULTS

Table 1 illustrates the levels of serum amylase in all the three groups. Three and six hours after AHNP induction, serum amylase increased significantly in AHNP group compared to that in sham operation group. But there was no significant difference between GdCl₃ pretreatment group and AHNP group 3 and 6 h after AHNP induction.

Table 2 shows the influence of GdCl₃ on hepatic functions of experimental animals. No changes were found in GdCl₃-treated animals and the values of ALT and AST in GdCl₃ control group were similar to those in sham group measured at all time points.

Serum TNF- α and IL-1 in AHNP group were significantly increased compared to those measured in sham group at all time points (P<0.01). In GdCl₃ pretreatment group, 3 and 6 h after AHNP induction, serum TNF- α and IL-1 were significantly decreased (P<0.01, Table 3).

Table 4 shows the MPO activity of lung injury in

Table 3 Serum TNF- α and IL-1 in all experimental groups (mean \pm SD)

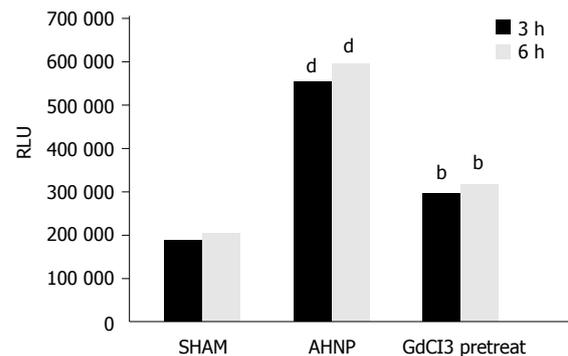
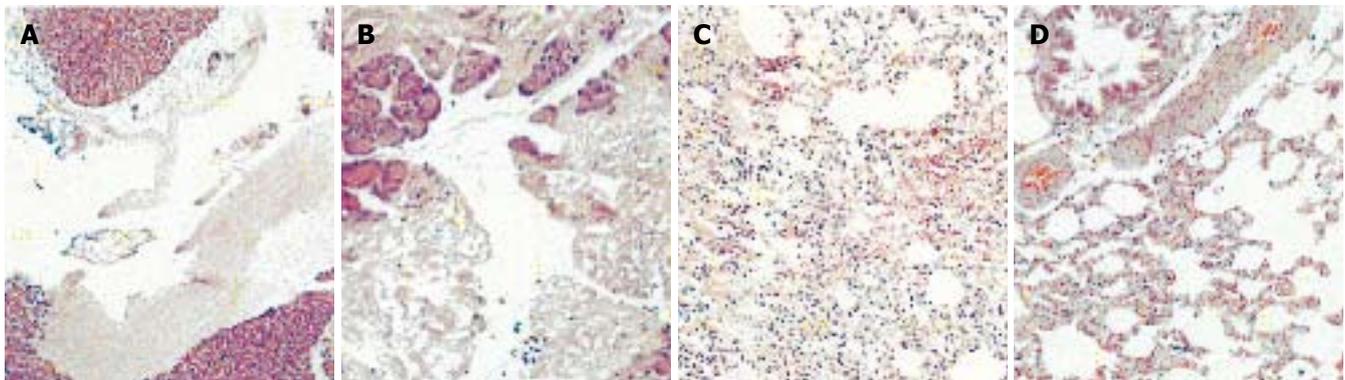
| Group | n | TNF- α (pg/mL) | IL-1 (μ g/L) |
|--------------------------------|---|------------------------------|------------------------------|
| 3 h | | | |
| Sham | 6 | 32.0 \pm 8.6 | 0.29 \pm 0.08 |
| AHNP | 6 | 113.9 \pm 18.5 | 0.81 \pm 0.11 |
| GdCl ₃ pretreatment | 6 | 71.1 \pm 14.3 ^b | 0.49 \pm 0.12 ^b |
| 6 h | | | |
| Sham | 6 | 31.9 \pm 8.2 | 0.28 \pm 0.07 |
| AHNP | 6 | 126.3 \pm 12.6 | 0.91 \pm 0.12 |
| GdCl ₃ pretreatment | 6 | 78.0 \pm 17.2 ^b | 0.53 \pm 0.13 ^b |

^bP<0.01 vs AHNP group.**Table 5 Histopathologic scores of lung tissue in all groups (mean \pm SD)**

| Group | Lung histopathologic scores | |
|--------------------------------|------------------------------|------------------------------|
| | 3 h (n = 6) | 6 h (n = 6) |
| Sham | 0.17 \pm 0.04 | 0.17 \pm 0.04 |
| AHNP | 2.50 \pm 0.55 ^b | 2.67 \pm 0.52 ^b |
| GdCl ₃ pretreatment | 1.87 \pm 0.41 ^a | 2.00 \pm 0.38 ^a |

^aP<0.05 vs AHNP group; ^bP<0.01 vs sham operation group.**Table 4 Lung tissue MPO level in all groups (mU/mg prot, mean \pm SD)**

| Group | MPO | |
|--------------------------------|------------------------------|------------------------------|
| | 3 h (n = 6) | 6 h (n = 6) |
| Sham | 0.51 \pm 0.13 | 0.50 \pm 0.17 |
| AHNP | 1.59 \pm 0.26 | 1.79 \pm 0.23 |
| GdCl ₃ pretreatment | 0.94 \pm 0.15 ^b | 1.02 \pm 0.19 ^b |

^bP<0.01 vs AHNP group.**Figure 1** NF- κ B activity of alveolar macrophages in BALF of all groups. ^bP<0.01 vs AHNP group, ^aP<0.01 vs sham operation group.**Figure 2** Histopathological changes in different groups. **A:** Extensive necrosis, intense edema and inflammatory infiltrate in AHNP group (100 \times); **B:** extensive necrosis and intense edema in GdCl₃ pretreatment group (100 \times); **C:** diffuse alveolar blood stasis and heavy infiltration of inflammatory cells (100 \times) in AHNP group; **D:** mild edema of the alveolar walls and mild alveolar blood stasis with slight infiltration of neutrophils in GdCl₃ pretreatment group (100 \times).

all the groups. The MPO levels in AHNP group were significantly higher than those in sham operation group (P <0.01). In GdCl₃ pretreatment group, 3 and 6 h after AHNP induction, the lung MPO levels were significantly decreased (P <0.01).

Figure 1 shows that the NF- κ B activity of alveolar macrophages in AHNP group was significantly higher than that in sham operation group (P <0.01) and was significantly decreased in GdCl₃ pretreatment group (P <0.01).

Histopathological study of the pancreas, 3 and 6 h after AHNP induction (Figure 2A) revealed extensive necrosis of pancreatic tissue, intense edema, and inflammatory infiltrate. The necrosis of pancreatic tissue and edema in GdCl₃ pretreatment group were similar to those in AHNP group (Figure 2B). The sham operation group was normal.

Diffuse alveolar blood stasis, intense alveolar septum

swelling and heavy infiltration of inflammatory cells mostly neutrophils were found in the lung tissue of AHNP group (Figure 2C). The mean histopathologic scores of AHNP group were significantly higher than those in sham operation group (P <0.01, Table 5). In GdCl₃ pretreatment group, the major histopathological findings were mild edema of the alveolar walls and mild alveolar blood stasis with slight infiltration of neutrophils (Figure 2D). The mean histopathologic scores of GdCl₃ pretreatment group decreased significantly compared to those of AHNP group (P <0.05).

DISCUSSION

Acute hemorrhagic necrotizing pancreatitis (AHNP) is a potentially fatal disease with a morbidity and mortality rate of approximately 30%. Acute lung injury (ALI)

is a common complication of AHNP, but the events that link AHNP and pulmonary damage are not fully understood. Many factors, such as oxygen free radicals, platelet activating factor, phospholipase A₂ (PLA₂), cyclooxygenase-2 (COX-2), cytokines and arachidonic acid metabolites are related to AHNP and ALI^[10-13]. Pancreatic proteolytic enzymes or activated PLA₂ released into the circulatory system determines the development of lung injury^[14]. Furthermore, other mediators in lung tissue such as platelet activating factor, arachidonic acid metabolites can stimulate inflammatory cell activation^[15,16]. Also, interaction of polymorphonuclear granulocytes, endothelium, and endothelium-derived mediators seems to be important to amplify lung damage^[17]. Recently, Cheng *et al*^[18,19] noted that activation of alveolar macrophages may play an important role in lung injury associated with AHNP, and that TNF- α and nitric oxide (NO) secreted by alveolar macrophages are increased significantly in rats with AHNP. Bhatia *et al*^[20] reported that inhibition of the production of hydrogen sulfide (H₂S) can significantly reduce the severity of cerulein-induced pancreatitis and associated-lung injury, suggesting an important proinflammatory role in regulating the severity of pancreatitis and associated-lung injury.

In recent years, some researchers found that the liver, especially KCs, might play a vital role in ALI caused by other different factors. Okutan *et al*^[21] reported that KCs blocked by GdCl₃ can attenuate lung damage caused by aortic ischemia reperfusion and malondialdehyde (MDA) level, an indicator of free radical generation and MPO activity, an indirect evidence of neutrophil infiltration in lung injury are decreased significantly^[21]. Although there is no evidence that GdCl₃ can suppress the function of neutrophils, it was reported that GdCl₃ can suppress the accumulation of neutrophils and alveolar macrophages^[22]. Feng *et al*^[23] investigated the role of KCs in the pathogenesis of ALI during acute obstructive cholangitis (AOC) and found that the phagocytic function of KCs is damaged in ALI induced by AOC.

KCs, the resident macrophages in the liver, are the major component of mononuclear phagocytic system (MPS). These macrophages make up 90% of the MPS and have abundant cytoplasm where abundant ribosome and phagosomes are located. These typical structures are associated with their functions. It was reported that KCs are responsible for the increased levels of TNF, IL-1, IL-6 in trauma, hemorrhagic shock and resuscitation. Decreasing the number or functional ability of KCs can lead to decreased levels of inflammatory cytokines as seen in the models of liver resection and sepsis^[24,25].

Also, KCs are regarded as the predominant source of inflammatory cytokines in AHNP at present^[26,27]. Closa *et al*^[4,5] performed an end-to-side portacaval shunt before AHNP induction in rats and found that portacaval shunting appears to exert a profound effect on ameliorating the inflammatory infiltrate. It is suggested that almost all the pancreatic enzymes and mediators released from the pancreas into the plasma during AHNP pass through the liver before their dilution in the systemic circulation, indicating that this step is a determinant in the development of the lung injury response. These

observations point to the key role of liver as a triggering mechanism for inflammatory processes in the lung as a consequence of AHNP. Moreover, activation of hepatic inflammatory cells, especially KCs, plays a key role in the development of lung injury^[4,5].

To improve our understanding of the role of KCs in AHNP-ALI, GdCl₃ was given 24 h prior to AHNP model induction to eliminate KCs in the present study. Moreover, the dose of GdCl₃ used in our study was 10 mg/Kg because administration of GdCl₃ at a dose 10 mg/kg can block the phagocytic activity of KCs completely^[28]. In GdCl₃ pretreatment group, the levels of MPO in lung tissue and the levels of TNF- α , IL-1 in serum all decreased significantly compared to those in AHNP group. NF- κ B activation of alveolar macrophages was also attenuated significantly in GdCl₃ pretreatment group compared to that in AHNP group. All these data suggest that GdCl₃ can ameliorate AHNP-ALI significantly and these results are in accordance with those reported by Folch *et al*^[29]. It was reported that lung injury associated with acute necrotic pancreatitis (ANP) is ameliorated by GdCl₃ through inducing apoptosis of alveolar macrophages of ANP^[30].

In our study, the serum amylase level did not decrease and the injury of pancreas was not prevented in GdCl₃ pretreatment group, suggesting that GdCl₃ cannot prevent lung injury by ameliorating pancreatic injury.

Using GdCl₃ as a tool to investigate the role of KCs is more reasonable than portacaval shunting operation which appears to exert a profound effect on animal's systemic circulation. Serum AST and ALT estimation suggests that GdCl₃ has no harmful effects on hepatic functions.

In conclusion, KCs play a vital role in AHNP and ALI.

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BASIC RESEARCH

Portal vein embolization induces compensatory hypertrophy of remnant liver

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Abstract

AIM: To evaluate the effectiveness and safety of different portal vein branch embolization agents in inducing compensatory hypertrophy of the remnant liver and to offer a theoretic basis for clinical portal vein branch embolization.

METHODS: Forty-one adult dogs were included in the experiment and divided into four groups. Five dogs served as a control group, 12 as a gelfoam group, 12 as a coil-gelfoam group and 12 as an absolute ethanol group. Left portal vein embolization was performed in each group. The results from the embolization in each group using different embolic agents were compared. The safety of portal vein embolization (PVE) was evaluated by liver function test, computed tomography (CT) and digital subtraction angiography (DSA) of liver and portal veins. Statistical test of variance was performed to analyze the results.

RESULTS: Gelfoam used for PVE was inefficient in recanalization of portal vein branch 4 wk after the procedure. The liver volume in groups of coil-gelfoam and absolute ethanol increased 25.1% and 33.18%, respectively. There was no evidence of recanalization of embolized portal vein, hepatic dysfunction, and portal hypertension in coil-gelfoam group and absolute ethanol group.

CONCLUSION: Portal vein branch embolization using absolute ethanol and coil-gelfoam could induce atrophy of the embolized lobes and compensatory hypertrophy of the remnant liver. Gelfoam is an inefficient agent.

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Key words: Portal vein embolization; Interventional

therapy; Liver; Dog

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INTRODUCTION

Primary carcinoma of the liver is one of the common malignant tumors in our country. Hepatectomy remains one of the most important therapies. However, only 15-30% of diagnosed patients are suitable for hepatectomy. Although extended hepatic resection is an effective therapy, partial excision of normal and functional liver is unavoidable in this surgery. If partial excision resects a certain amount of liver, patients especially those with liver cirrhosis, would suffer from postoperative liver failure. Although limiting resection area can reduce the possibility of complications, it increases the incidence of postoperative recurrence and metastasis. These problems can be effectively solved by artificially inducing hyperplasia of normal liver tissue preoperatively. Therefore, preoperative portal vein embolization (PPVE) is an effective procedure to settle this contradiction^[1-5].

In 1920, Roust Larimore discovered that ligation of rabbits' portal vein branch could contribute to the atrophy of pathological lobe and hyperplasia of non-pathological one. In 1975, Honjo *et al*^[6] tried portal vein ligation in liver cancer patients not fit for hepatic resection, which resulted in atrophy of the ligated lobe of portal vein and its tumor and hyperplasia of non ligated lobe. In 1980s, Makuuchi *et al*^[7] found that if the portal vein of one lobe is embolized by tumor thrombi, hyperplasia usually occurs in the other lobe of the liver, and meanwhile, patients enjoy better recovery after hepatectomy because unexpected rising of portal vein pressure could be avoided. These findings have led to the exploration of preoperative PVE prior to extensive liver hepatic resection. PVE in clinical practice^[1,2,8,9] and experimental studies^[10-13] has achieved success. Since foreign emboli materials are expensive and difficult to obtain, it is essential to screen for easily-obtained, convenient, cheap, and safe embolic agents to effectively induce compensatory hyperplasia. The purpose of our study was to evaluate the effectiveness and safety of three convenient and cheap agents in inducing compensatory hypertrophy of liver.

MATERIALS AND METHODS

Experimental animals

Forty-one healthy adult dogs weighing 16.2±4.2 kg (range, 12.0-20.0 kg) without obvious growth (to avoid sudden changes of weight during the process of experiment, which would affect liver volume and influence experimental results), were included in the study. All dogs were provided by Experimental Animal Center of Union Hospital attached to Fujian Medical University. Before and after the experiment, they were fed with same standard forage. Five served as the control group and the remaining 36 were divided into 3 groups at random, 12 in each group. Gelfoam, coil-gelfoam and absolute ethanol were applied to these 3 experimental groups for portal vein embolization.

Methods

A dog with jejunitis on operative day was given an intravenous injection of 10% pentobarbital sodium into the hind legs to induce general anesthesia. Then, the dog underwent helical CT scanning to conduct pre and post contrast-enhanced CT study of its liver. The liver volume was measured automatically by a computer with the help of SOMATOM PLUS4 spiral CT scanner (Siemens Co.). The dog was transferred into the interventional operating room and cut open for catheter placement of portal vein under general anesthesia. After a 5F Cobra catheter was inserted into the trunk of portal vein, 76% angiografin was injected at the speed of 8-10 mL/s through the catheter to study the branch pattern of portal vein. Because the right lobe was relatively fixed, left portal vein was chosen as a target vessel for embolization. Then, the catheter was inserted into the left portal vein and saline or an embolic agent was injected into the appropriate position in different experimental groups. Control group: 30-50 mL saline was injected through the catheter. Gelfoam group: gelfoam was cut into strips of 10 mm×1.0 mm, which were inserted into the embolized portal vein through the catheter with saline until complete embolization was achieved. Embolization procedure was stopped when the embolized portal vein was completely embolized and the distal portal vein branch was not opacified. The number of gelfoams used ranged between 40-60 strips. Coil-gelfoam group: gelfoam was used to embolize smaller branches of the left portal vein, then coils were used to embolize the trunk of the left portal vein until satisfactory results were achieved. The number of coils ranged between 3-5. Absolute ethanol group: Five milliliters of absolute ethanol was slowly injected through the catheter. After several minutes, an additional amount of absolute ethanol was provided based on the embolization situation of portal vein in order to achieve the result of complete embolization. The amount of absolute ethanol was generally 0.5-1.0 mL/kg. After embolization, 9-12 mL angiografin was injected through the catheter at a speed of 6-8 mL/s to carry out angiography of the portal vein, which would allow us to understand its iconography expression after embolization. At the same time, we measured the pressure of main portal vein, and the radial lines of the nonembolized portal veins including the right portal vein and main portal vein.

Table 1 Changes of the right lobe volume in gelfoam group (cm³)

| No | Pre-embolization | 4W | 6W | 8W |
|---------|------------------|------------|------------|------------|
| Dog A1 | 250 | 265 | 260 | 263 |
| Dog A2 | 338 | 340 | 334 | 348 |
| Dog A3 | 380 | 395 | 370 | 398 |
| Dog A4 | 273 | 279 | 277 | 280 |
| Dog A5 | 370 | 368 | 373 | 378 |
| Dog A6 | 320 | 318 | 334 | 332 |
| Dog A7 | 229 | 245 | 230 | 237 |
| Dog A8 | 342 | 365 | 344 | 377 |
| Dog A9 | 360 | 353 | 373 | 374 |
| Dog A10 | 362 | 363 | 370 | 368 |
| Mean±SD | 318±18.2 | 347.6±21.4 | 321.7±17.7 | 331.9±19.4 |

After embolization, all dogs were regularly fed. Angiography was performed 4 and 8 wk after embolization, transcatheter portal vein angiography was undertaken to understand the iconography expression of portal vein branches, and then diameter of the nonembolized portal vein was measured. Changes of portal vein pressure were monitored 4 and 8 wk after embolization, transcatheter portal vein angiography was performed to measure the portal vein pressure. Changes of liver function were detected before and after embolization, alanine aminotransferase (ALT) was assayed every 2 d until liver function became normal. Changes of liver volume were determined 4, 6, and 8 wk after embolization, liver CT scanning was performed to measure the volume of the nonembolized lobe. Dogs were killed 8 wk after embolization, pathology observation was implemented of liver tissues of the embolized and nonembolized lobes.

Statistical analysis

SPSS11.0 software package was adopted to carry out statistical analysis of experimental results.

RESULTS

Control group

During the period of embolization, all the five dogs survived. Iconography expression was the same as before, suggesting that no blood vessel was embolized. Meanwhile, there were no changes of portal vein pressure, volume of the right lobe, liver function, and pathology before and after saline injection.

Gelfoam group

One dog died of respiratory failure, another dog died of acute liver failure due to gelfoam back-flow which led to extensive embolization of portal vein branches in liver, and 10 dogs survived. Post embolization angiography showed that the left branch of portal vein was completely embolized, while the right branch was not obstructed, the right portal vein and main portal vein were dilated compared to pre-embolization. Four weeks after embolization the embolized left portal vein had blood flow (Figure 1). The portal vein pressure was 94.1±1.8 mm H₂O, 123.2±2.8 mm H₂O before embolization, and 94.7±2.0 mm H₂O, 95.8±6.6 mm H₂O after embolization, respectively ($P>0.05$). ALT value obviously increased 2 d after embolization ($P<0.01$), but decreased subsequently

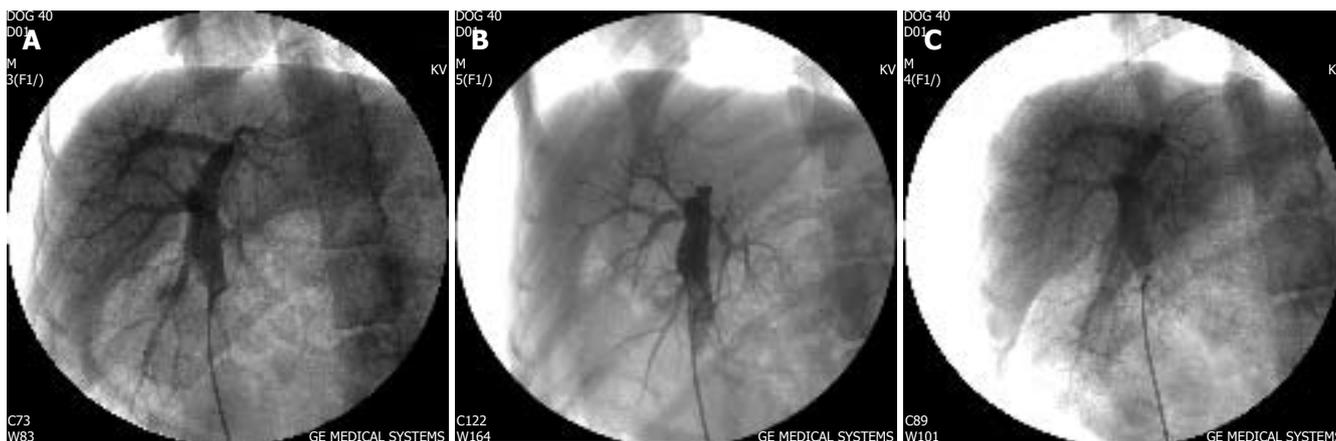


Figure 1 Portal vein angiography before (A) and after (B) embolization as well as 4 wk (C) after embolization with gelfoam.

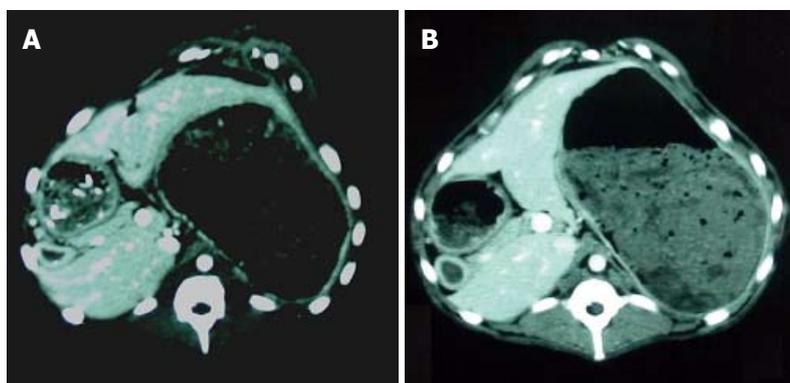


Figure 2 No obvious changes in volume of the right lobe before (A) and after (B) embolization.

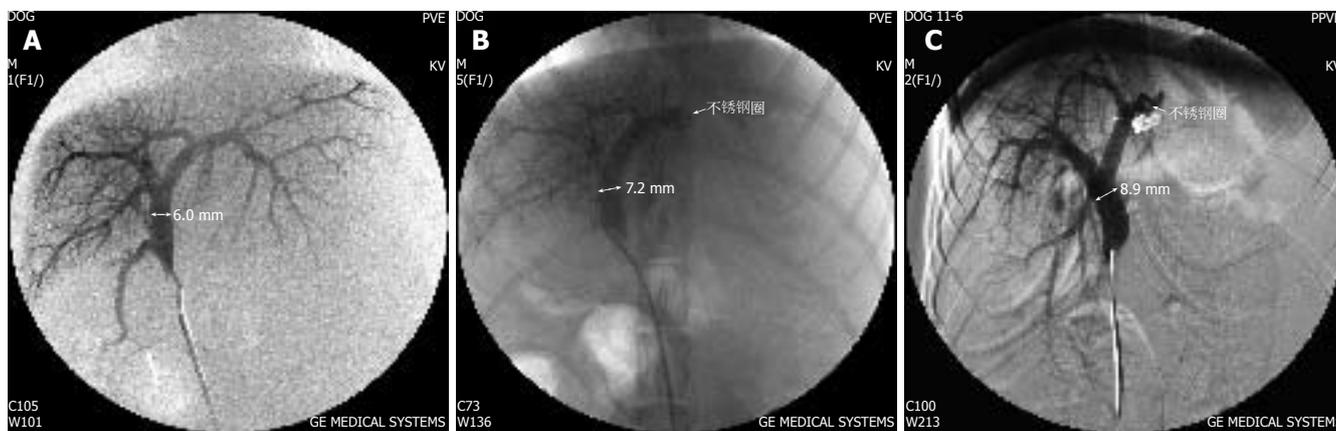


Figure 3 Portal vein angiography before (A) and after (B) embolization as well as 8 wk (C) after embolization with coil-gelfoam.

and then became normal in the following 3 d. No obvious changes were found in the volume of the right lobe before and after embolization (Table 1). Contrast-enhanced CT scanning revealed that the left branch of portal vein was recanalized (Figure 2). Ten dogs were killed 8 wk after embolization, no obvious differences were found between the embolized and nonembolized lobes.

Coil-gelfoam group

One dog died of respiratory failure due to narcosis, the rest had successful surgery (91.7%). After embolization, the left branch was completely embolized while the

right branch was patent. Compared to pre-embolization situations, the main portal vein and right portal vein were expanded to a certain extent (Figure 3). The portal vein pressure was 96.5 ± 5.1 mm H₂O, 133.9 ± 10.4 mm H₂O before embolization and 97.0 ± 6.3 mm H₂O, 97.6 ± 4.7 mm H₂O after embolization. ALT in experimental dogs increased 2 d after embolization ($P < 0.01$), and returned to the pre-embolization level in the following 3 d ($P > 0.05$). The right lobes of all the survived dogs after embolization were expanded to varying extent. The liver volume was $344.9 + 38.3$, $374.8 + 43.0$ before embolization and $431.5 + 50.9$, $434.0 + 50.4$ after embolization (Table

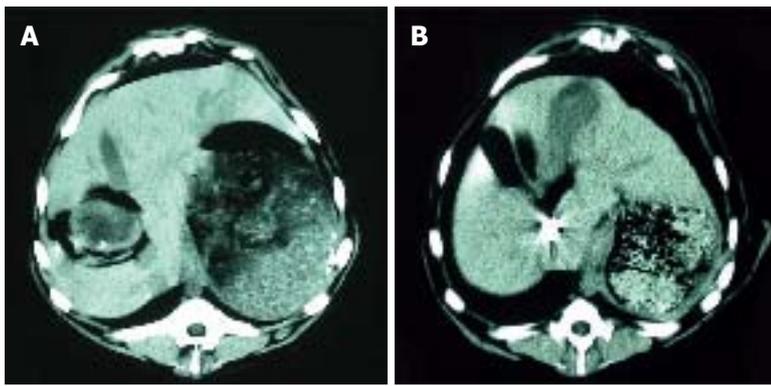


Figure 4 Changes in volume of the right lobe before (A) and after (B) embolization.

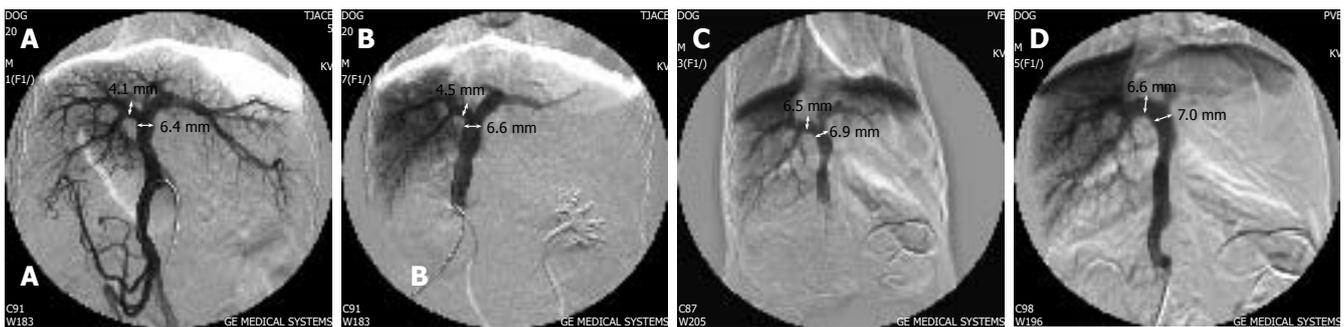


Figure 5 Portal vein angiography before (A) and after (B) embolization as well as 4 wk (C) and 8 wk (D) after embolization with absolute ethanol.

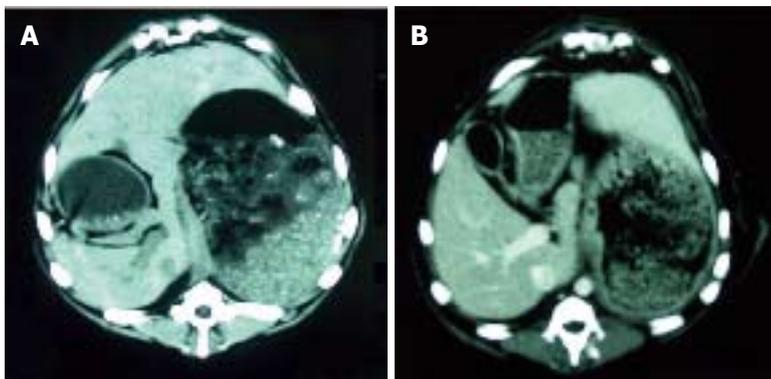


Figure 6 Obvious changes in volume of the right lobe before (A) and after (B) embolization.

Table 2 Changes of the right lobe volume in coil-gelfoam group (cm³)

| No | Pre-embolization | 4W | 6W | 8W |
|---------|------------------|------------|------------|------------|
| Dog B1 | 380 | 414 | 474 | 478 |
| Dog B2 | 298 | 325 | 369 | 370 |
| Dog B3 | 376 | 412 | 470 | 472 |
| Dog B4 | 332 | 362 | 417 | 420 |
| Dog B5 | 360 | 392 | 450 | 451 |
| Dog B6 | 294 | 310 | 363 | 365 |
| Dog B7 | 310 | 340 | 379 | 382 |
| Dog B8 | 388 | 426 | 491 | 495 |
| Dog B9 | 300 | 326 | 378 | 385 |
| Dog B10 | 376 | 413 | 473 | 474 |
| Dog B11 | 380 | 403 | 482 | 482 |
| Mean±SD | 344.9±38.3 | 374.8±43.0 | 431.5±50.9 | 434.0±50.4 |

Table 3 Changes of the right lobe volume in absolute ethanol group (cm³)

| No. | Pre-embolization | 4W | 6W | 8W |
|---------|------------------|------------|------------|------------|
| Dog C1 | 360 | 392 | 495 | 495 |
| Dog C2 | 272 | 301 | 363 | 363 |
| Dog C3 | 356 | 390 | 492 | 498 |
| Dog C4 | 319 | 350 | 438 | 437 |
| Dog C5 | 329 | 359 | 447 | 449 |
| Dog C6 | 326 | 357 | 430 | 430 |
| Dog C7 | 289 | 293 | 387 | 392 |
| Dog C8 | 328 | 352 | 437 | 439 |
| Dog C9 | 303 | 329 | 412 | 413 |
| Dog C10 | 400 | 409 | 470 | 473 |
| Mean±SD | 328.2±39.3 | 353.3±40.3 | 437.1±45.0 | 438.9±45.6 |

2). No further hyperplasia was found (Figure 4). The embolized lobe was dark red with a sharp edge, while the nonembolized lobe was slightly bright red and comparatively plump. Coils were surrounded by fibrin and

combined forming the tight emboli. Under microscope, the embolized lobe showed structural changes of hepatic lobules, atrophy and degeneration of liver cells, and congestion of blood vessel, while the nonembolized lobe

revealed cell hypertrophy and obvious hyperplasia.

Absolute ethanol group

One dog died during surgery and 1 dog died in 24 h after surgery due to respiratory failure and acute liver failure caused by the back-flow of absolute ethanol. The rest 10 dogs had successful surgery. The left branch of portal vein was completely embolized, while the right branch was patent. Meanwhile, the main portal vein and right portal vein compared to pre-embolization, were dilated to a certain degree. The left branch of portal vein was occluded without recanalization 8 wk after embolization, while blood flow was seen in the right portal vein and further dilatation was seen in the main portal vein and right portal vein (Figure 5). The portal vein pressure was 95.2 ± 5.2 mm H₂O, 125.2 ± 6.4 mm H₂O before embolization and 96.2 ± 5.2 mm H₂O, 95.8 ± 6.6 mm H₂O after embolization. ALT was 25.2 ± 1.2 , 62.2 ± 3.2 , 28.8 ± 1.6 , and 26.1 ± 1.3 IU/L after 1, 3, 5, and 7 d of embolization, respectively. ALT values increased by different degrees according to the different injection amount of absolute ethanol, decreased dramatically 3-5 d after embolization and then returned to the pre-embolization level after 1 wk. The volume of right lobe was 328.2 ± 37.1 , 353.2 ± 37.9 before embolization and 436.7 ± 42.5 , 438.9 ± 45.6 after embolization. No further liver hypertrophy was found (Figure 6). Experimental dogs were killed 8 wk after embolization for pathological observation. The embolized lobe was dark red with a sharp edge, while the nonembolized lobe was slightly bright red and comparatively plump. Under microscope, the embolized lobe showed structural changes of hepatic lobules, liver atrophy, degeneration of liver cells and congestion of fiber tissue, while the nonembolized lobe revealed cell hypertrophy and obvious hyperplasia (Table 3).

DISCUSSION

All our experimental groups with coil-gelfoam and absolute ethanol as embolic agents for left portal vein had right lobe hypertrophy, but the control group and gelfoam group failed to embolize the left portal vein, suggesting that there was no compensatory hypertrophy in the right lobe. The increasing blood flow to the nonembolized portal vein is one of the important factors for liver regeneration. After PVE, dilatation of the nonembolized portal vein branch with obviously increased blood flow could lead to hyperplasia of the nonembolized lobe. These changes were found in our study. Nutritious substances can stimulate regeneration of liver cells. As we know, portal vein blood contains nutritious substances which stimulate regeneration of liver cells. In clinical practice, patients with liver cirrhosis or portal hypertension after shunt operation would suffer from liver atrophy due to decreased blood flow of portal vein. Lindroos *et al*^[14] reported that hepatocyte growth factor (HGF) could increase 17 times after two-thirds of rat liver were resected and 13 times after being treated with carbon tetrachloride. Kinoshita *et al*^[15] reported that the amount of HGF mRNA in rat liver interstitial cells increased obviously after being treated with carbon tetrachloride, indicating that the increase of HGF can lead to the regeneration of liver cells.

The increase of portal vein pressure and abnormal liver function after PVE, were transient and returned to normal after 4 wk and 1 wk, respectively^[16], indicating that transient ischemia of liver portal vein branch has a limited impact on liver function. In our study, five dogs died of accidental extensive embolization of liver portal vein or respiratory failure related to narcosis overdose or absolute ethanol. If PVE is applied in clinical practice, overdose of narcosis can be avoided. The levels of ALT and total bilirubin increased after PVE and returned to normal in 1-2 wk^[17,18], demonstrating that the abnormal liver function secondary to PVE is transient and reversible, and does not result in permanent damage to liver function. The portal vein pressure of the three experimental groups was measured at different time points after embolization. We found that the portal vein pressure after embolization was usually higher after embolization, and then returned to normal within 4 wk. Our result is consistent with the study of Baere *et al*^[2]. Portal hypertension has not been found in long-term follow-up^[19].

All kinds of materials such as absolute ethanol, polydocanol, and gelfoam can be used for portal vein embolization^[20-22]. Park *et al*^[23] used percutaneous puncture of portal vein to inject Embol into the left branches of pigs' portal vein, and then killed these pigs at different time points to show the safety and effect of Embol. Ko *et al*^[24] employed Embol-78 to embolize portal vein of patients with hepatocellular carcinoma or with nonhepatocellular carcinoma to evaluate its effect and safety. Wu *et al*^[25] carried out selective embolization experiment of portal vein branches in SD rats with α -cyanoacrylate (DTH) and found compensatory hyperplasia of the nonembolized lobe, suggesting that DTH can effectively induce compensatory hypertrophy of nonembolized lobes. Brown *et al*^[26] adopted transcatheter portal vein operation to embolize portal vein branches of liver-cancer patients with polyvinyl alcohol particles (PVA). The above PVA embolization materials have many disadvantages, such as high price, deficient specifications and lack of supply. Gelfoam, coils and absolute ethanol with different characteristics have been used as embolic agents in our clinical practice. Gelfoam can last for a moderate length of time and is safe, non-poisonous and cheap. However, during the process of portal vein embolization, a large amount of gelfoam should be used to completely embolize distal branches of the portal vein, because it easily results in back-flow of gelfoam and accidental embolization of non-target portal vein. We also found that recanalization occurred in gelfoam-embolic portal vein, which failed to effectively induce compensatory hypertrophy of the nonembolized lobe. Therefore, it is not suitable and safe to use gelfoam as an embolic agent for PVE. On the other hand, after being inserted into the target blood vessel, coils lead to mechanical embolization and neointimal hyperplasia, forming permanent embolization. However, coils cannot embolize small blood vessels and easily result in collateral vessels, limiting its use as a PVE embolic agent for inducing hypertrophy of liver. Therefore, combination of gelfoam and coils is a kind of safe and effective mode to achieve complete embolization and to avoid displacement and accidental embolization. In addition, absolute ethanol

can instantly produce permanent embolization in target blood vessels without collateral vessel formation after embolization, thus preventing recanalization of blood vessels and achieving better embolized results. The above results show that absolute ethanol is better than coil-gelfoam for PVE. However, when absolute ethanol is used as an embolic agent, back-flow easily occurs. Once back-flow occurs, it results in serious consequences. It is safe and effective to settle back-flow of absolute ethanol using a balloon catheter^[27,28]. In absolute ethanol group, two dogs died of injection of large absolute ethanol doses, while the rest of the dogs which received an injection of small absolute ethanol doses survived. A larger amount of absolute ethanol can achieve good embolic effect, but death rate of experimental animals increases. A smaller amount of absolute ethanol cannot achieve satisfactory results. Thus, further research is needed to determine the safe amount of absolute ethanol injected. Absolute ethanol is a peripheral embolic agent. Compared to coil-gelfoam, it can reach distal branches of portal vein. After embolization, collateral vessels are difficult to form. At the same time, absolute ethanol exerts stronger influence on embolized portal vein branches and even smaller distal branches. Absolute ethanol causes certain damage to neointimal cells inside small branches of hepatic arteries and hepatic cells. The event enhances embolic effect, prevents recanalization of blood vessel, thus stimulating and inducing compensatory hypertrophy of nonembolized lobes more significantly.

In conclusion, gelfoam alone cannot effectively induce compensatory hypertrophy. Coil-gelfoam and absolute ethanol can be used as selective embolic agents to induce compensatory hypertrophy of liver, but absolute ethanol is better than coil-gelfoam for inducing compensatory hypertrophy. However, the safety of absolute ethanol is inferior to that of coil-gelfoam.

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Analysis of p53 and vascular endothelial growth factor expression in human gallbladder carcinoma for the determination of tumor vascularity

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Abstract

AIM: To examine the expression of p53 and vascular endothelial growth factor (VEGF) as well as microvessel count (MVC) and to investigate the role of VEGF as an angiogenic marker and the possible role of p53 in the regulation of angiogenesis in human gallbladder carcinoma.

METHODS: Surgically resected specimens of 49 gallbladder carcinomas were studied by immunohistochemical staining for p53 protein, VEGF, and factor VIII-related antigen. VEGF expression and mutant p53 expression were then correlated with Nevin stage, differentiation grade, MVC, and lymph node metastasis.

RESULTS: Positive p53 protein and VEGF expressions were found in 61.2% and 63.3% of tumors, respectively. p53 and VEGF staining status was identical in 55.1% of tumors. The Nevin staging of p53- or VEGF-positive tumors was significantly later than that of negative tumors. The MVC in p53- or VEGF-positive tumors was significantly higher than that in negative tumors, and MVC in both p53- and VEGF-negative tumors was significantly lower than that in the other subgroups.

CONCLUSION: Our findings suggest that p53-VEGF pathway can regulate tumor angiogenesis in human gallbladder carcinoma. Combined analysis of p53 and VEGF expression might be useful for predicting the tumor vascularity of gallbladder cancer.

INTRODUCTION

Angiogenesis refers to the formation of new blood vessels from a pre-existing vascular network. Adequate vascularization is critically required for the growth and metastasis of human solid tumors^[1-5]. Angiogenesis is influenced by multiple factors such as growth factors, extracellular matrix proteins, and cell adhesion molecules, as well as by the imbalance between angiogenesis-stimulating factors and inhibiting factors^[6-10]. One of the most important angiogenesis-stimulating factors is VEGF, a diffusible endothelial cell-specific mitogen that induces endothelial-cell proliferation and increases vascular permeability. There is evidence that it may promote a degradative environment that facilitates migration of endothelial cells by the conduction of plasminogen activators and collagenase^[11]. VEGF plays a major role in regulating angiogenesis. Most tumors produce high levels of VEGF. Neutralization of VEGF leads to a marked inhibition of angiogenesis and tumor growth^[12-15].

However, there is little information about the genetic changes associated with angiogenesis. So far, most studies have stressed the role of oncogene activation and tumor suppressor gene (TSG) inactivation in promoting aberrant tumor-cell proliferation. Although it remains unclear whether any of these genetic alterations can trigger the disruption of control of angiogenesis, some *in vitro* studies have demonstrated the important role played by the p53 tumor suppressor gene in controlling tumor angiogenesis^[16,17]. In the present study, we have examined p53 and VEGF expressions as well as microvessel count (MVC) in human gallbladder carcinoma tissues to investigate the involvement of the p53 gene in regulation of tumor angiogenesis and its clinical significance.

Table 1 Nevin staging system for gallbladder cancer^[18]

| Stage | Definitions |
|-------|---|
| 1 | Tumor invades mucosa only |
| 2 | Tumor invades muscularis and mucosa |
| 3 | Tumor invades subserosa, muscularis, and mucosa |
| 4 | Tumor invades all layers of gallbladder wall plus cystic lymph node |
| 5 | Tumor extension into liver bed or distant spread |

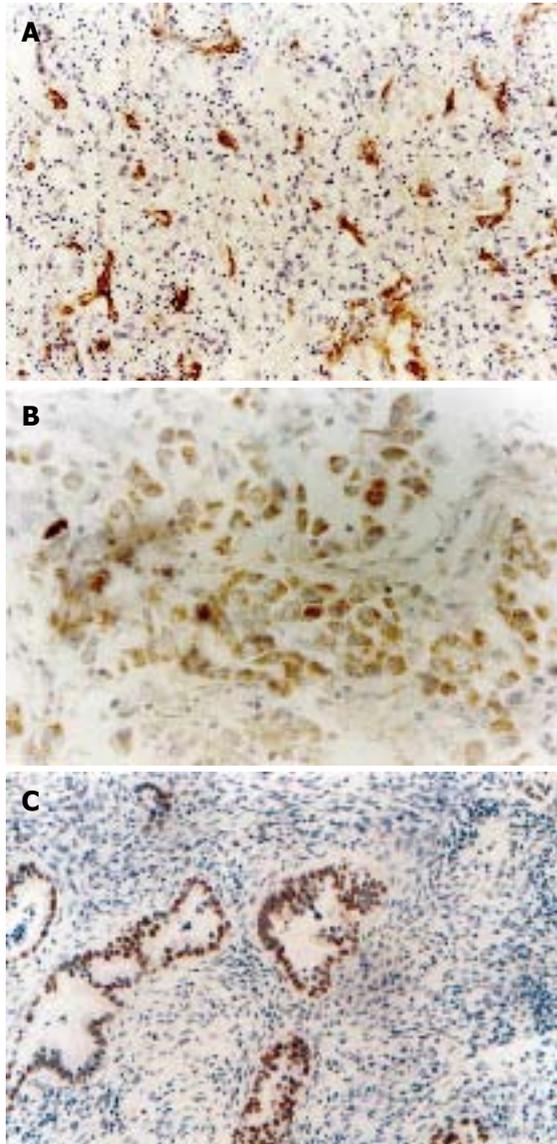


Figure 1 Microvessel distribution (A) and expression of VEGF (B) and p53 (C) in gallbladder carcinoma tissue.

MATERIALS AND METHODS

Clinical materials

Forty-nine histologically proven gallbladder carcinomas were selected. All patients were surgically treated at the Department of General Surgery of the First and Second Hospitals affiliated to China Medical University, Shenyang, China, but did not receive chemotherapy or anti-angiogenesis therapy before surgery. The cases included 24 males and 25 females. The average age of the males and females was 62 years and 55 years, respectively.

Six cases had papillary adenocarcinoma (12.2%), 43 cases had tubular adenocarcinoma (87.8%), 22 cases had well-differentiated tumor (44.9%), 17 cases had moderately differentiated tumor (34.7%), 10 cases had poorly differentiated tumor (20.4%). Nevin stage (Table 1) was determined based on clinical materials: 19 cases of S1, S2, and S3, and 30 cases of S4 and S5. Twenty-seven cases (55.1%) had lymph node metastasis (+), 22 cases (44.9%) had no lymph node metastasis (-). In each case, all available sections stained with hematoxylin and eosin were reviewed.

Immunohistochemical study of p53 and VEGF

Four micrometer thick sections from the formalin-fixed and paraffin-embedded tissues were placed on the poly-L-lysine-coated slides for immunohistochemistry.

Immunohistochemical staining was performed by the streptavidin-biotin method. In brief, sections were deparaffinized and incubated with 3% hydrogen peroxide for 20 min to block endogenous peroxidase activity. The sections were treated twice with microwave at 500 W for 5 min each time in 10 mmol/L sodium citrate (pH 6.0). After washing with PBS, the sections were incubated in 10% normal rabbit or goat serum for 20 min to reduce non-specific antibody binding. The antibodies used were mouse monoclonal antibody (MAb) against human p53 protein (Maxin-Bio Co., Fuzhou, China) in 1:100 dilution at 4 °C overnight, and a rabbit polyclonal antibody against human VEGF (A-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 1:50 dilution at 4 °C overnight. After washing thrice with PBS, the sections were incubated with biotinylated rabbit anti-mouse or goat anti-rabbit immunoglobulin G (Maxin-Bio Co., Fuzhou, China) for 30 min, washed thrice again with PBS, treated with streptavidin-peroxidase reagent for 30 min and then washed thrice with PBS again. Finally, the specimens were incubated in PBS containing diaminobenzidine and 1% hydrogen peroxide for 5 min and counterstained with hematoxylin. PBS was substituted for each primary antibody as negative control. Slides were examined by two investigators without the knowledge of the corresponding clinicopathologic data. p53 immunoreactivity was assessed as being positive only when tumors exhibited intense nuclear staining, and reactivity was categorized into negative expression (less than 10% positive tumor cells) and positive expression (at least 10% positive tumor cells). Immunostaining for VEGF was considered positive when unequivocal staining of cell membrane or cytoplasm was observed in more than 10% of tumor cells.

Microvessel staining and counting

Microvessel staining and counting were performed as described previously^[19]. Briefly, intratumoral microvessels were highlighted by immunostaining with a mouse MAb against factor VIII-related antigen (F-VIII RAg) (Maxin-Bio Co., Fuzhou, China) in 1:100 dilution and incubated at 4 °C overnight, after pre-digestion with 0.1% (v/v) trypsin at 37 °C for 20 min. Any single brown-stained cell or cluster of endothelial cells that was clearly separated from adjacent vessels, tumor cells and other connective tissues was considered as a microvessel. The stained sections were screened at ×40 fields to identify the regions of the high-

Table 2 Clinicopathologic characteristics of MVC in gallbladder carcinoma (mean ± SD)

| Characteristics | n | MVC |
|-----------------------|----|--------------------|
| Tumor differentiation | | |
| Good | 22 | 30±11 |
| Moderate-poor | 27 | 38±11 ^a |
| Nevin staging | | |
| S1, S2, S3 | 19 | 26±11 |
| S4, S5 | 30 | 40±8 ^b |
| Lymph node metastasis | | |
| Yes | 27 | 40±10 |
| No | 22 | 28±11 ^d |

^aP<0.05 vs good group; ^bP<0.01 vs "S1, S2, S3" group; ^dP<0.01 vs yes group.

Table 3 Clinicopathologic characteristics of VEGF expression in gallbladder carcinoma

| Characteristics | n | + | % |
|-----------------------|----|----|-------------------|
| Tumor differentiation | | | |
| Good | 22 | 15 | 68.2 |
| Moderate-poor | 27 | 16 | 59.3 |
| Nevin staging | | | |
| S1, S2, S3 | 19 | 8 | 42.1 |
| S4, S5 | 30 | 23 | 76.7 ^a |
| Lymph node metastasis | | | |
| Yes | 27 | 21 | 77.8 |
| No | 22 | 12 | 54.5 ^c |

^aP<0.05 vs "S1, S2, S3" group; ^cP<0.05 vs yes group.

Table 4 Clinicopathologic characteristics of mutant p53 expression in gallbladder carcinoma

| Characteristics | n | + | % |
|-----------------------|----|----|-------------------|
| Tumor differentiation | | | |
| Good | 22 | 13 | 59.1 |
| Moderate-poor | 27 | 17 | 63.0 |
| Nevin staging | | | |
| S1, S2, S3 | 19 | 9 | 47.4 |
| S4, S5 | 30 | 21 | 70.0 ^a |
| Lymph node metastasis | | | |
| Yes | 27 | 20 | 74.1 |
| No | 22 | 10 | 45.5 ^c |

^aP<0.05 vs "S1, S2, S3" group; ^cP<0.05 vs yes group.

Table 5 Relationship between MVC and presence of p53 and VEGF (mean ± SD, n (%))

| | Patients, VEGF(+) | MVC | |
|--------|---------------------|----------------------------------|----------------------------------|
| | | VEGF(-) | Total |
| p53(+) | 27 (55.1%) 41±9 | 3 (6.1%) 35±10 | 30 (61.2%) 36±10 |
| p53(-) | 4 (8.2%) 36±12 | 15 (30.6%) 28±13 ^b | 19 (38.8%) 30±12 ^c |
| Total | 31 (63.3%) 37±11 | 18 (36.7%) 30±12 ^c | 49 (100%) |

^aP<0.05 vs others, ^bP<0.05 vs "VEGF(+)" group, ^cP<0.05 vs "p53(+)" group.

est vascular density within the tumor. Vessels were counted in the five regions of the highest vascular density at ×200 fields (Olympus BH-2 microscope, 0.74 mm² per field). MVC was the mean number of vessels in these areas.

Statistical analysis

The relationship between p53 or VEGF expression and MVC was evaluated by *t*-test, and the relationship between p53 and VEGF expression and various clinicopathologic factors was examined by the χ^2 test. *P*<0.05 was considered statistically significant.

RESULTS

Expression of VEGF, p53, and MVC

The microvessels in malignant tissues were heterogeneously distributed. These neovascular areas occurred anywhere within the tumor but most frequently at the tumor margins (Figure 1A). The immunoreactive regions of VEGF were located in cytoplasm or membranes of the gallbladder carcinoma cells (Figure 1B). The immunoreactive regions of mutant p53 were located in the nuclei of the gallbladder carcinoma cells (Figure 1C).

Clinicopathologic characteristics of MVC, VEGF, and p53 expressions

The average MVC in 49 cases of gallbladder carcinoma was (35±12)/HP. MVC was markedly higher in cases of Nevin stage S4-S5 than in those of S1-S3 (*P*<0.01). MVC in moderate-poor differentiation group was higher than that in good differentiation group (*P*<0.05). MVC

in patients with lymph node metastasis was significantly higher than that in those without lymph node metastasis (*P*<0.01, Table 2).

The positive rate of VEGF expression was 63.3% in these 49 cases and was higher in cases of Nevin stage S4-S5 (76.7%) than in those of S1-S2 (42.1%) (*P*<0.05). The positive rate of VEGF expression was not correlated with tumor differentiation (*P*>0.05). With regard to the association with lymph node metastases, the positive rate of VEGF expression was significantly higher in patients with metastasis than in those without metastasis (*P*<0.05, Table 3).

The positive rate of mutant p53 expression was 61.2% in these 49 cases and was lower in cases of Nevin stage S1-S3 (47.4%) than in those of S4-S5 (70.0%), the difference was statistically significant (*P*<0.05). The positive rate of mutant p53 expression was not correlated with tumor differentiation (*P*>0.05). With regard to the association with lymph node metastases, the positive rate of p53 expression was significantly higher in patients with metastasis than in those without metastasis (*P*<0.05, Table 4).

Correlation between p53, VEGF, and MVC

Table 5 shows the relationships between MVC and the presence of p53 and VEGF. Staining status was identical in 27 of 49 tumors (55.1%), and a significant (*P*<0.05) association between p53 and VEGF expression was demonstrated. The MVC in tumors that were p53 or VEGF positive was significantly (*P*<0.05) higher than that in p53- or VEGF-negative tumors. Moreover, the

mean MVC in tumors of all the subgroups that were both p53 and VEGF positive was the highest, while the mean MVC was significantly lower in patients with tumors that were both p53 and VEGF negative than in all the other subgroups.

DISCUSSION

In 1971, Folkman proposed that tumor growth depends on angiogenesis. Now there is considerable indirect and direct evidence that tumor growth is dependent on angiogenesis. Numerous studies showed that neovascularization is closely associated with growth, invasion, metastasis, staging and prognosis of tumors^[7,20-23]. Our study also indicated that angiogenesis was correlated with the Nevin staging and tumor differentiation. The higher the staging is, the poorer the differentiation and the higher the level of MVC are in gallbladder carcinoma. Since MVC is of prognostic value, we investigated whether this holds true for VEGF expression as well. Our findings demonstrate that both VEGF expression and MVC are significantly elevated in patients with disease progression and depend on each other. Tumors with established poor outcome (staging, MVC) are likely to produce higher levels of VEGF, suggesting that VEGF significantly contributes to the poor outcome in these patients.

The most common genetic alteration in various cancers is loss of the p53 tumor suppressor gene function. P53 protein has various important functions in cellular integration, including cell growth control, response to DNA damage, checkpoint mechanisms during the cell cycle, regulation of transcription and control of genomic stability. It is suggested that p53 protein may play a role in suppressing angiogenesis^[24-27]. In addition, it was reported that a pathway from p53 regulating VEGF induces angiogenesis in cellular transfection models^[16]. In the present study, we explored the possibility of a relationship between aberrant p53 and VEGF expression and tumor angiogenesis in gallbladder carcinoma. We observed that VEGF-positive tumors had significantly more microvessels than VEGF-negative tumors. Our findings agree with other studies^[13,28,29], suggesting that VEGF-induced tumor angiogenesis plays an important role not only in tumor progression but also in metastasis. Although little is known concerning the VEGF regulatory pathways, Kieser *et al.*^[16] reported that mutant-type p53 might stimulate VEGF expression. In the present study, p53 and VEGF expression status coincided in 27 of 49 (55.1%) tumors, and a significant association was found between the two factors. Moreover, MVC was the highest in p53 and VEGF positive tumors and the lowest in p53 and VEGF negative tumors. These results suggest not only the existence of a p53-VEGF regulatory pathway in gallbladder carcinomas, but also a possible role of such a pathway in regulating tumor angiogenesis in this type of cancer. These further suggest that tumors that have lost p53 suppressor function not only permit uncontrolled cell growth, but also possibly produce a suitable environment for hematogenous metastasis by stimulating VEGF-induced angiogenesis in the primary site of carcinoma. In contrast, tumors with preserved p53 function and angiogenic inhibitory pathway

are less possible to progress, and the p53 gene may therefore contribute to better prognosis.

In conclusion, our results demonstrate that abnormal p53 accumulation is closely associated with VEGF expression and tumor vascularity in human gallbladder carcinoma. P53 plays a critical role in suppressing tumor growth by regulating tumor angiogenesis. Clinical application of combined analysis of p53 and VEGF expression may be useful for predicting the clinical staging, occurrence of metastasis in patients with this disease. More prospective studies should be made to prove our findings.

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BASIC RESEARCH

Effect of genistein on voltage-gated potassium channels in guinea pig proximal colon smooth muscle cells

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Abstract

AIM: To investigate the action of genistein (GST), a broad spectrum tyrosine kinase inhibitor, on voltage-gated potassium channels in guinea pig proximal colon smooth muscle cells.

METHODS: Smooth muscle cells in guinea pig proximal colon were enzymatically isolated. Nystatin-perforated whole cell patch clamp technique was used to record potassium currents including fast transient outward current (I_{Kto}) and delayed rectifier current (I_{Kdr}), two of which were isolated pharmacologically with 10 mmol/L tetraethylammonium or 5 mmol/L 4-aminopyridine. Contamination of calcium-dependent potassium currents was minimized with no calcium and 0.2 mmol/L CdCl₂ in an external solution.

RESULTS: GST (10-100 μmol/L) reversibly and dose-dependently reduced the peak amplitude of I_{Kto} with an IC₅₀ value of 22.0±6.9 μmol/L. To a lesser extent, I_{Kdr} was also inhibited in both peak current and sustained current. GST could not totally block the outward potassium current as a fraction of the outward potassium current, which was insensitive to GST. GST had no effect on the steady-state activation ($n=6$) and inactivation kinetics ($n=6$) of I_{Kto} . Sodium orthovanadate (1 mmol/L), a potent inhibitor of tyrosine phosphatase, significantly inhibited GST-induced inhibition ($P<0.05$).

CONCLUSION: GST can dose-dependently and reversibly block voltage-gated potassium channels in guinea pig proximal colon smooth muscle cells.

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Key words: Protein tyrosine kinase; Patch-clamp technique; Genistein; Voltage-gated potassium channel; Colon; Smooth muscle cells

Li SY, Huang BB, Ouyang S. Effect of genistein on voltage-gated potassium channels in guinea pig proximal colon

INTRODUCTION

Ion channels are targets of many intracellular signaling pathways, including protein phosphorylation and dephosphorylation. These processes can modify channel activity and dramatically alter the electrophysiological properties of both excitable and nonexcitable cells^[1]. In addition to the extensive information available about the regulation of ion channels by serine-threonine kinases, an emerging body of evidence suggests that channels are also regulated by phosphorylation on tyrosine residues, such as ligand-gated channels AChR^[2], NMDA receptor^[3], potassium channels^[4,5] as well as calcium channels^[6,7]. GST, a specific inhibitor of tyrosine kinases, inhibits visceral smooth muscle contraction induced by tyrosine phosphatase inhibitor vanadate^[8], angiotensin II^[9] and carbachol^[10] via tyrosine kinase-mediated process. Potassium channels, which are substrates for protein phosphorylation and dephosphorylation, control the contraction of gastrointestinal smooth muscles by setting resting potential and influencing slow waves and action potential configuration^[11]. However, little is known of the modulation of potassium channels in gastrointestinal smooth muscle cells via tyrosine kinase pathway. This study was to investigate the effects and mechanisms of GST on voltage-gated potassium channels (Kv) in smooth muscle cells of guinea pig proximal colon.

MATERIALS AND METHODS

Cell dissociation

Smooth muscle cells were enzymatically isolated using modified procedures as previously described^[12]. Briefly, male guinea pigs (200-350 g) were killed by cervical dislocation and proximal colon about 2 cm aboral to cecum was rapidly excised. Under anatomical microscope, smooth muscle strips were dissected out, cut into small pieces and incubated for 30 min in low calcium solution containing 10 mmol/L HEPES, 135 mmol/L NaCl, 6 mmol/L KCl, 0.05 mmol/L CaCl₂, 1.2 mmol/L MgCl₂, 10 mmol/L Glucose, pH 7.4. The pieces of muscles were then transferred to low calcium solution containing

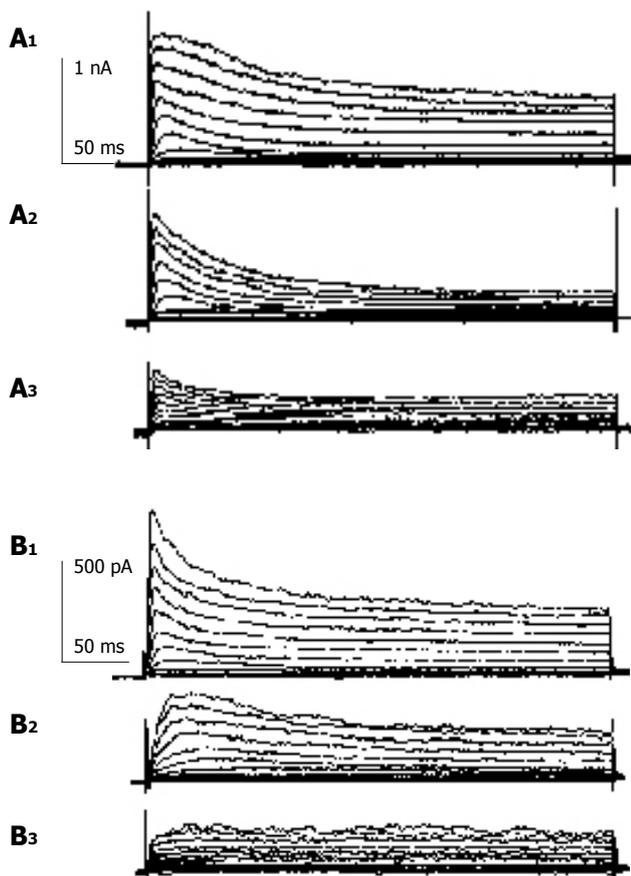


Figure 1 Two components of voltage-gated potassium currents in guinea pig colon smooth muscle cells and effect of GST. **A₁-A₃**: Currents recorded every 10 s between -80 and +50 mV then back to -40 mV with holding potential -80 mV in the control (**A₁**), in the presence of 10 mmol/L TEA (**A₂**) and TEA and 30 μmol/L GST (**A₃**). **B**: Currents recorded every 10 s between -80 and +50 mV with holding potential -80 mV in the control; **B₁**: current in the presence of 5 mmol/L 4-AP developed slowly and showed slower inactivation over 400 ms; **B₃**: effect of 50 μmol/L GST in the presence of 5 mmol/L 4-AP.

3 g/L papain, 2 g/L DTT, 2 g/L bovine serum albumin. Tissues were incubated at 36 °C in enzyme solution for 15 min and then suspended in enzyme-free low calcium solution. Tissue pieces were gently agitated to create a cell suspension. Dispersed cells were stored at 4 °C for later use. Experiments were performed at 20-22 °C within 10 h.

Perforated whole cell voltage clamp recording

Relaxed single colon smooth muscle cells with smooth appearance and spindle shape observed under an inverted microscope (IX70, Olympus) were used. Myocytes were perfused with Ca²⁺-free cell bath solution in a self-made small volume chamber in which solution could be exchanged in 30 s. The composition of bath solution was the same as that of low calcium solution except for exclusion of calcium and inclusion of 0.2 mmol/L CdCl₂ to minimize the contamination of calcium-dependent potassium currents.

Patch clamp micropipettes were pulled with a programmable puller (P-97, Sutter Instruments) and their tips were fire polished (CPM-2, ALA Co.). The pipette resistance was 3-5 MΩ. Currents were amplified with Axopatch 200B (Axon Instruments). Data were filtered at 1 kHz and analyzed with pClamp software (version 8.2).

Nystatin-perforated whole cell patch clamp technique was used to record voltage-gated potassium currents. Nystatin was dissolved in DMSO at a concentration of 25 g/L, and then added to the internal pipette solution to yield a final nystatin concentration of 100 mg/L. The internal solution contained 10 mmol/L HEPES, 110 mmol/L glucoante (potassium salt), 30 mmol/L KCl, 10 mmol/L NaCl, 2 mmol/L MgCl₂, pH 7.2. After giga seals were obtained, access resistance was monitored for 10 min to allow the drop of access resistance (average 17.7 ± 5.7 MΩ, $n=28$) and then compensated at 70%. Macroscopic current values were normalized for cell capacitance as whole cell current densities (pA/pF). The average cell capacitance was 41.3 ± 7.5 pF ($n=28$ cells).

Drugs and chemicals

Papain, 4-aminopyridine (4-AP), nystatin, tetraethylammonium (TEA), sodium orthovanadate (VAN), genistein (GST) were purchased from Sigma. GST was prepared as a 50 mmol/L stock solution in DMSO and stored at -20 °C. DTT (BBI), HEPES, DMSO, bovine serum albumin were from Shanghai Sangon Biological Engineering Technology and Services Company.

Statistical analysis

Data were expressed as mean ± SD. Differences in the data were evaluated by paired or independent *t*-test when appropriate. $P < 0.05$ was considered statistically significant. Software Microcal Origin 5.0 was used for statistical analysis and graph plotting.

RESULTS

Potassium currents in single smooth muscle cells

Outward potassium currents are mainly composed of voltage-gated potassium current and calcium-dependent potassium current. Under the conditions of our recordings (no added Ca²⁺ and 0.2 mmol/L CdCl₂ in the bath solution), contamination of currents through calcium-dependent potassium currents was minimized.

Currents were evoked using standard stimulus protocol, i.e., the membrane potential was stepped for 400 ms from a holding potential of -80 mV to test potentials between -80 and +50 mV in 10 mV increments. Depolarization to potentials positive to -40 mV activated non-linear, time-dependent outward currents which could be divided into transient outward potassium current (I_{kto}) and delayed rectifier potassium current (I_{kdr}) as shown in Figure 1. I_{kto} was sensitive to millimolar concentration of 4-AP and insensitive to TEA. I_{kdr} was on the contrary. When 10 mmol/L TEA was externally applied, early peak current of transient outward current (peak current among the first 50 ms of test pulse, I_{peak}) was only slightly decreased, but the quasi steady state current (average current from 350 to 400 ms after test pulse onset, I_{ss}) was blocked -60% (Figure 1A). When 5 mmol/L 4-AP was externally applied, I_{peak} was much reduced (Figure 1B) and the time to half-maximum current at test potential was significantly increased (data not shown), I_{ss} was little affected. Detailed description of voltage-dependent potassium current of guinea pig proximal colon smooth muscle cells could

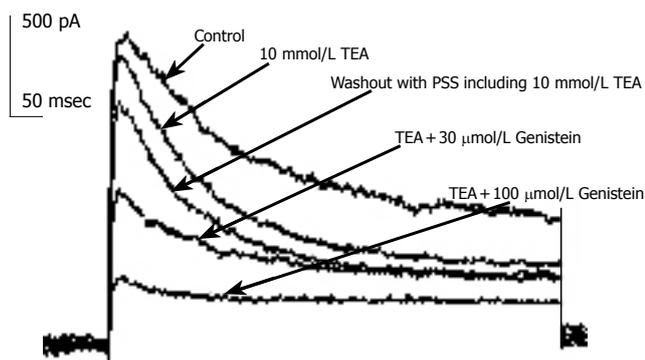


Figure 2 Concentration-dependent inhibition of transient outward potassium currents by genistein in guinea pig colon smooth muscle cells. Currents were elicited by depolarization to +50 mV for 400 ms then back to -40 mV from holding potential of -80 mV. Washout was performed for about 2 min.

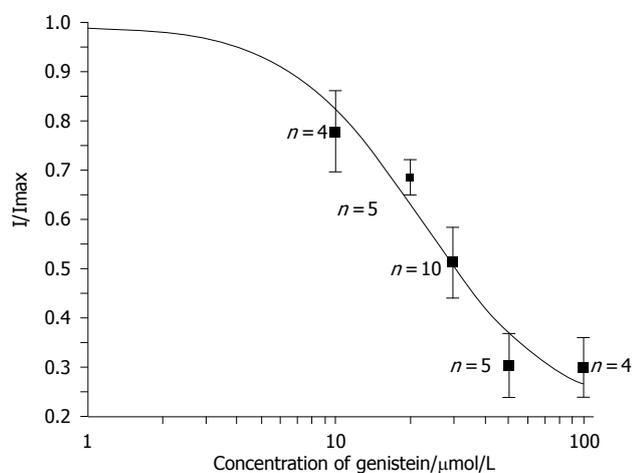


Figure 3 Average concentration-dependent transient outward potassium currents inhibited by genistein. Peak currents were measured and normalized by control current amplitude. The smooth line represents the best fitting with Hill equation. Data are expressed as mean±SE. The number of cells used in each concentration is indicated besides the error bars.

be seen elsewhere^[13], which agrees well with our study. According to the different sensitivity to TEA and 4-AP, we isolated these two kinds of current pharmacologically to study the effect of GST independently.

Concentration-dependent inhibition of GST on I_{Kto}

Currents recorded were mainly I_{Kto} when 10 mmol/L TEA was externally applied to bath solution. Repetitive single current traces were elicited for 400 ms from a holding potential of -80 mV to test potentials +50 mV in every 20s until stable currents were recorded. I_{peak} of K_{to} decreased progressively and reached a stable state in about 1-2 min after the external perfusion of GST. GST (10-100 μ mol/L) induced a reduction in I_{peak} , with little effect on the steady state potassium current. The inhibitory effects of GST were reversible (Figure 2). Perfusion of calcium-free saline including TEA could restore the amplitude of currents to about 80% of control in 2 min (data not shown).

GST could not totally block the outward potassium currents even when 100 μ mol/L GST was applied. Since no significant difference in the inhibitory effect was

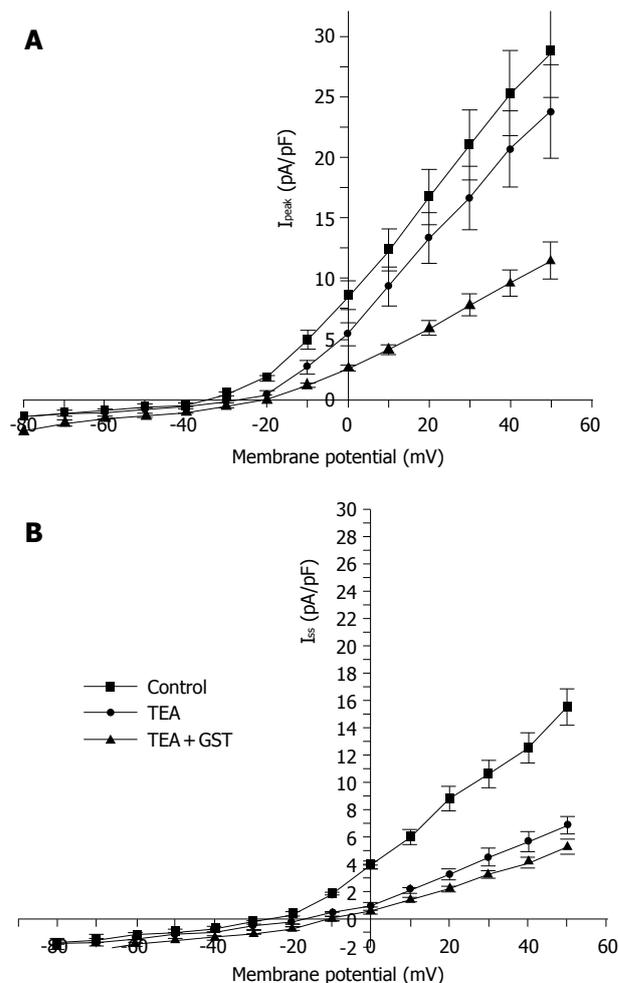


Figure 4 Effect of genistein (30 μ mol/L) on current-voltage relationship of transient outward potassium current (I_{Kto}) in guinea pig proximal colon smooth muscle cells in the presence of TEA (10 mmol/L) and Cd^{2+} (0.2 mmol/L). **A:** I - V curves of peak current density of voltage-gated potassium channels under control condition (solid square), in the presence of 10 mmol/L TEA (solid circle) as well as 10 mmol/L TEA and 30 μ mol/L genistein (solid triangle); **B:** I - V curves of steady state current density of voltage-gated potassium channels under control condition, in the presence of 10 mmol/L TEA as well as 10 mmol/L TEA and genistein (30 μ mol/L). $n=6$ cells.

found between 50 ($n=10$) and 100 ($n=5$) μ mol/L GST, a fraction of the outward potassium current was insensitive to GST.

Early peak transient outward potassium current of K_{to} measured at +50 mV test pulse from a holding potential of -80 mV was used as an index of inhibition, which was plotted as a function of GST concentration (10-100 μ mol/L). The data were fitted with $I_{GST}/I_{control} = 1 / \{1 + (IC_{50}/[D])^n\}$, where $[D]$ is the concentration of GST used, IC_{50} is the concentration at half maximal inhibition, and n is the Hill coefficient. Figure 3 shows that the effect of GST on I_{peak} of K_{to} was concentration-dependent, with an IC_{50} of 22.0 ± 6.9 μ mol/L.

Effect of GST on current-voltage relationship (I - V curves) of I_{Kto}

Currents were evoked using standard stimulus protocol. The membrane potential was stepped for 400 ms from a holding potential of -80 mV to test potentials between -80

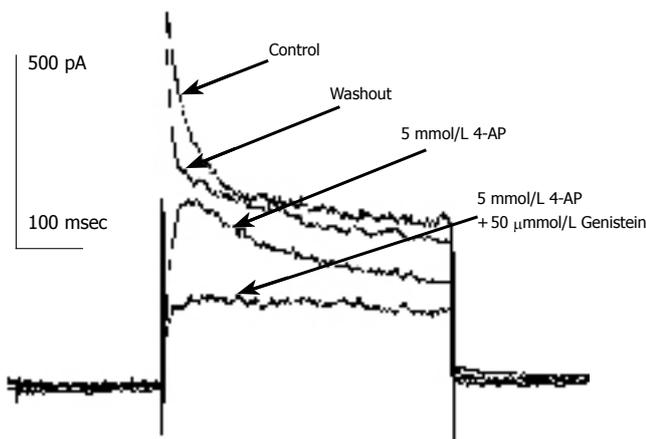


Figure 5 Effect of genistein on delayed rectifier potassium currents in the presence of 4-AP in guinea pig colon smooth muscle cells. Currents were elicited by depolarization to +50 mV for 400 ms then back to -40 mV from holding potential of -80 mV. Washout was performed for about 2 min.

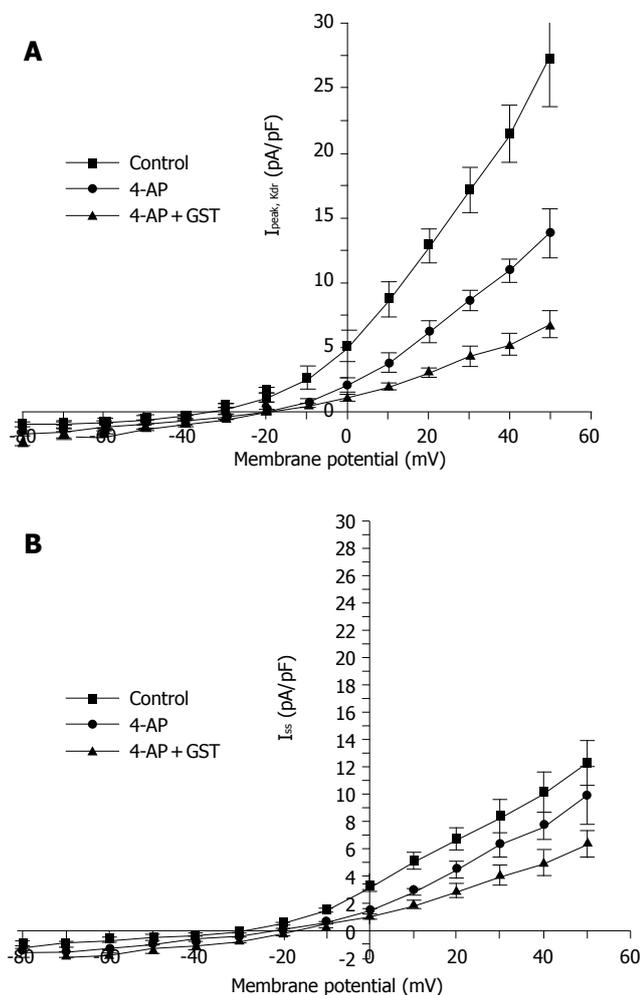


Figure 6 Effect of genistein (50 $\mu\text{mol/L}$) on current-voltage relationship of delayed rectifier potassium current (I_{Kdr}) in guinea pig proximal colon smooth muscle cells in the presence of 4-AP (5 mmol/L) and Cd^{2+} (0.2 mmol/L). **A:** $I-V$ curves of peak current density of voltage-gated potassium channels under control condition, in the presence of 5 mmol/L 4-AP as well as 5 mmol/L 4-AP and 50 $\mu\text{mol/L}$ genistein; **B:** $I-V$ curves of steady state current density of voltage-gated potassium channels under control condition, in the presence of 5 mmol/L 4-AP as well as 5 mmol/L 4-AP and 50 $\mu\text{mol/L}$ genistein. $n=4$ cells.

and +50 mV in 10 mV increments. The interval between two pulses was set at 10 s to allow inactivated conductance to recover. I_{peak} and I_{ss} were measured at test pulses from -80 to +50 mV and converted into current densities, which were plotted as a function of test pulse.

Figure 1 shows that in the presence of 10 mmol/L TEA, 30 $\mu\text{mol/L}$ GST significantly blocked I_{peak} ($P<0.05$, $n=6$ cells) and only slightly reduced I_{ss} . Figure 4 shows the averaged $I-V$ curves of I_{Kto} inhibited by GST. The inhibition showed no voltage dependence.

Effect of GST on I_{Kdr}

Currents recorded in the presence of external 5 mmol/L 4-AP were mainly delayed rectifier potassium currents. Repetitive single current traces were elicited for 400 ms from a holding potential of -80 mV to test potentials +50 mV in every 20 s until stable currents were recorded. In the presence of 5 mmol/L 4-AP, 50 $\mu\text{mol/L}$ GST reduced peak current and steady state current with a fractional inhibition of current f [$f=(1-I_{\text{GST}}/I_{\text{drug}})\times 100\%$] of $52\pm 8\%$ ($P<0.05$, $n=4$) and $33\pm 2\%$ ($P<0.05$, $n=4$), respectively. The inhibitory effects of GST were reversible. Perfusion of calcium-free saline could restore the amplitude of currents in 2 min (data not shown). Details are shown in Figure 5.

Effect of GST on $I-V$ curves of I_{Kdr}

The same experimental protocol as that of the study on the effect of GST on I_{Kto} was used. Figure 1B shows that in the presence of 5 mmol/L 4-AP, 50 $\mu\text{mol/L}$ GST significantly blocked I_{peak} as well as I_{ss} ($P<0.05$, $n=4$ cells). Figure 6 shows the averaged $I-V$ curves of I_{Kdr} inhibited by GST. The inhibition showed no voltage dependence, and 50 $\mu\text{mol/L}$ GST had a greater effect on I_{Kto} than on I_{Kdr} ($P<0.05$).

Effect of GST on steady state activation kinetics of I_{Kto}

The activation curves of I_{Kto} were derived from $I-V$ curves of I_{Kto} . G_{Kto} was calculated by dividing the initial peak current value by their driving force and plotted as a function of membrane potential. The activation curves were fitted with a Boltzmann function $G/G_{\text{max}}=1/\{1+\exp[(V-V_{\text{h}})]/\epsilon\}$, where V is membrane potential, V_{h} is the half-maximal activation voltage, and ϵ is the slope constant (mV). GST had no significant effect on steady state activation kinetics of I_{Kto} . The value of half-maximal activation voltage V_{h} was 3.3 ± 1.3 and 3.5 ± 1.4 mV, respectively in the absence and presence of 30 $\mu\text{mol/L}$ LGST, the slope constant ϵ was 12.9 ± 1.0 and 13.3 ± 1.1 mV, respectively ($n=6$, Figure 7).

Effect of GST on steady state inactivation kinetics of I_{Kto}

Classic double pulse protocol was used to determine the voltage dependence of inactivation of I_{Kto} . Currents were elicited by a depolarizing pulse of +50 mV with 1 s preconditioning pulses from -80 to 20 mV by increment of 10 mV. The interval between two sweeps was set at 20s. A plot of normalized peak current (I/I_{max}) as a function of preconditioning potential was fitted with a Boltzmann function: $I/I_{\text{max}}=1/\{1+\exp[(V_{\text{h}}-V)]/\epsilon\}$, where V is the

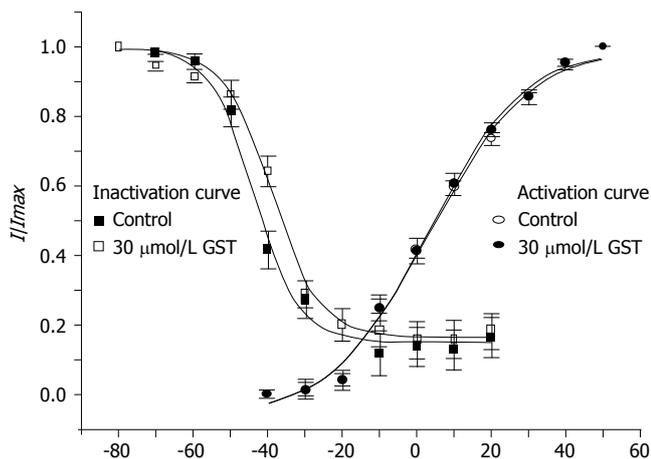


Figure 7 Effect of GST on steady state activation and inactivation of transient outward potassium currents. Inactivation is shown as a plot of normalized peak current as a function of conditional potential from -80 to +20 mV. For voltage dependence of activation, normalized currents were calculated by dividing peak outward currents by their driving force and plotted against potential. Smooth curves were fitted with Boltzmann equation which yields values of half-maximally activated or inactivated voltage (V_h) and slope constant (K).

membrane potential, V_h is the half-maximal inactivation voltage, and k is the slope constant (mV). GST had no significant effect on steady state inactivation kinetics of I_{Kto} . The values of half-maximal inactivation voltage V_h were -43.4 ± 0.7 and -39.2 ± 0.8 mV, respectively in the absence and presence of $30 \mu\text{mol/L}$ GST, the slope constant k was 5.8 ± 0.6 and 6.5 ± 0.7 mV, respectively ($n=6$, Figure 7).

VAN blocked inhibition of GST on I_{Kto}

Inhibition of phosphatase-mediated tyrosine dephosphorylation would be expected to block current inhibition by GST, since dephosphorylation in the presence of GST would require ongoing phosphatase activity^[14]. At commonly used bath concentrations of 0.1-10 mmol/L, inhibitor of tyrosine phosphatase orthovanadate could antagonize various GST-induced cellular responses such as blocking the inhibition of $I_{Ca,L}$ by PTK inhibitors^[15]. Solely applied VAN had minor stimulating but no significant effect on I_{Kto} ($n=7$). In the presence of 1 mmol/L VAN, the fractional inhibition of current f of $30 \mu\text{mol/L}$ GST significantly decreased to $28 \pm 6\%$ (Figure 1) compared with $49 \pm 6\%$ of solely applied GST ($n=10$). The rapid onset and offset responses to GST did not change (Figure 8).

DISCUSSION

At least four types of potassium channel have been identified in excitable gastrointestinal smooth muscle cells, such as voltage-dependent outward potassium channel, calcium-dependent potassium channel, ATP-sensitive potassium channel, and inward rectifier potassium channel. A given smooth muscle cell can express several families of potassium channels and several members of a single family of channels. This complexity is necessary for the control of smooth muscle function^[11]. K_v channels are of particular importance in the regulation of colonic smooth muscle electrical activity because they provide

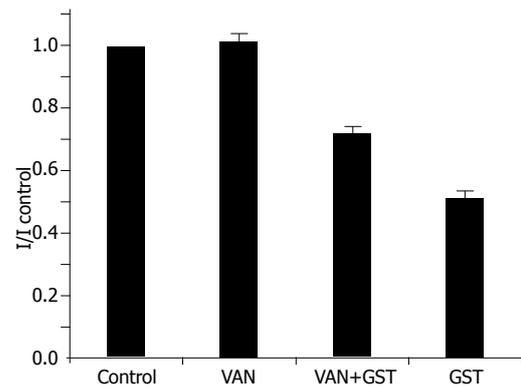


Figure 8 Orthovanadate antagonizes the inhibition of transient outward potassium currents by genistein.

outward currents over the voltage range in which these tissues operate^[11]. K_v shapes the action potential (AP) by controlling its repolarization phase and determines the membrane potential and duration of the interspike interval. Delayed rectifier potassium channels keep single AP short and permit high-frequency trains of APs. Transient outward potassium channels help a cell fire at low frequency and promote broadening of APs during repetitive activity^[16]. In murine colon, application of 4-AP to intact preparations can abolish the quiescent periods between slow waves and induce a slight depolarization^[11]. I_{Kto} is fully inactivated during the upstroke depolarization^[17]. In this study, GST could block transient outward potassium currents to depolarize membrane potential as well as delayed rectifier potassium currents to induce slight depolarization^[11], thus modulating the contraction of smooth muscle.

Protein tyrosine kinase activity is a major signaling mechanism in regulating long-term processes such as cell growth, division, and metabolism^[18]. PTK signaling is also important in regulating ion channel conductance. GST, a natural isoflavone which is abundant in soybean, is a specific inhibitor of tyrosine specific kinases by competing with ATP to form the nonproductive enzyme-substrate complexes^[19]. In this study, GST concentration - dependently and reversibly blocked voltage-gated potassium currents. Though it was reported that GST has no specific effects such as direct interaction with potassium channels^[14,20,21], orthovanadate could antagonize the blockage of GST on I_{Kto} , suggesting that although direct blockage of potassium channels by a mechanism unrelated to PTK inhibition could not be entirely excluded in this study, GST blocks the transient outward potassium channels partly via PTK pathway.

There are some differences in GST action on ion channel currents and contraction of different types of smooth muscle. GST can inhibit visceral smooth muscle contraction induced by vanadate^[8], angiotensin II^[9] and carbachol^[10] as well as nifedipine-sensitive calcium currents in rabbit colon myocytes^[22]. GST also can inhibit apamin-sensitive relaxation of the longitudinal muscle in rat distal colon induced by pituitary adenylate cyclase activating peptide^[23] as well as potassium channels^[4,5]. Besides species and tissue differences, it is possible that multiple receptor

and nonreceptor PTKs are involved in the regulation of smooth muscle contraction. Further studies are required to identify the physiological role of PTKs in gastrointestinal motility.

To check whether GST could affect the biophysical kinetics of voltage-dependent potassium channels, we examined the steady state activation and inactivation kinetics in the absence and presence of 30 $\mu\text{mol/L}$ GST. Peretz *et al.*^[4] found that GST affects potassium gating properties of Schwann cells such as a positive shift in voltage dependence of activation (by +30 mV) and a decrease in steepness of activation gating. In our experiment, no significant effect of GST on gating properties of potassium channels was observed. Whether species or cell type difference contributes to the variation needs further investigation.

In conclusion, GST concentration dependently and reversibly inhibits transient outward potassium currents.

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CLINICAL RESEARCH

Long-term outcome of endoscopic metallic stenting for benign biliary stenosis associated with chronic pancreatitis

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promising treatment options for bile duct stenosis associated with CP, provided the patients are closely followed up; thus setting a system for their prompt management on emergency is desirable.

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Key words: Chronic pancreatitis; Biliary stricture; Metallic stent; Long-term outcome

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Abstract

AIM: Endoscopic metal stenting (EMS) offers good results in short to medium term follow-up for bile duct stenosis associated with chronic pancreatitis (CP); however, longer follow-up is needed to determine if EMS has the potential to become the treatment of first choice.

METHODS: EMS was performed in eight patients with severe common bile duct stenosis due to CP. After the resolution of cholestasis by endoscopic naso-biliary drainage three patients were subjected to EMS while, the other five underwent EMS following plastic tube stenting. The patients were followed up for more than 5 years through periodical laboratory tests and imaging techniques.

RESULTS: EMS was successfully performed in all the patients. Two patients died due to causes unrelated to the procedure: one with an acute myocardial infarction and the other with maxillary carcinoma at 2.8 and 5.5 years after EMS, respectively. One patient died with cholangitis because of EMS clogging 3.6 years after EMS. None of these three patients had showed symptoms of cholestasis during the follow-up period. Two patients developed choledocholithiasis and two suffered from duodenal ulcers due to dislodgement of the stent between 4.8 and 7.3 years after stenting; however, they were successfully treated endoscopically. Thus, five of eight patients are alive at present after a mean follow-up period of 7.4 years.

CONCLUSION: EMS is evidently one of the very

INTRODUCTION

Chronic pancreatitis (CP) is reported to become complicated with bile duct stenosis in about 5-40% of the patients^[1,2]. The severity of the stenosis varies; however, radical treatment is needed for serious cases presenting persistent cholestasis, jaundice or cholangitis. Moreover, it has been shown that long-standing biliary stenosis, even if mild or moderate, often causes liver damage^[3,4]. Half of these patients will present liver fibrosis by the time of decompression^[5].

Non-surgical treatment has been reported to be comparable with surgery, with lower morbidity and mortality. Especially, endoscopic biliary drainage (ERBD) using a plastic tube stent has been adopted as the first-line treatment, and there are many reports concerning the usefulness of ERBD^[5,6]. Although plastic stents offer satisfactory short-term drainage, medium to long-term results have been disappointing because of stent clogging or migration^[6,7], and surgical treatment is chosen for a permanent palliation. However, surgical treatment is associated with important morbidity^[8].

ERBD using a metallic stent (EMS) is useful for malignant biliary stricture since a relatively long-term non-surgical palliation can be attained^[9]. In contrast, EMS for a benign biliary stricture is still controversial^[10,11]. Furthermore, there have been few reports on EMS for biliary stenosis due to CP. Deviere *et al* reported favorable

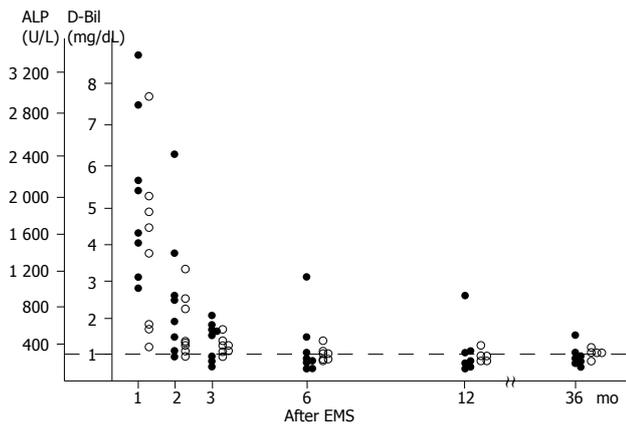


Figure 1 Plotting of the serum alkaline phosphatase concentration (●) and conjugated bilirubin (○) of each patient during 3 yr after EMS. The broken line indicates the upper limit of normal.

Table 1 Clinical background of the patients

| Patient number | Age (yr) | Sex | Cause of CP | Underlying diseases | Jaundice | Cholangitis |
|----------------|----------|------|-------------|---------------------|----------|-------------|
| 1 | 78 | Male | Alcohol | Angina Pectoris | + | + |
| 2 | 53 | Male | Alcohol | DM | - | + |
| 3 | 42 | Male | Alcohol | DM, Colon cancer | + | - |
| 4 | 43 | Male | Alcohol | | - | - |
| 5 | 77 | Male | Alcohol | | + | + |
| 6 | 53 | Male | Alcohol | DM | + | + |
| 7 | 70 | Male | Alcohol | DM | + | + |
| 8 | 67 | Male | Alcohol | DM | + | + |

CP: Chronic pancreatitis; DM: Diabetes mellitus.

results with the self-expandable metal mesh stent for biliary obstruction due to CP, but the long-term results have not been reported^[12]. In this study, we intended to clarify the outcome of patients with biliary stenosis due to CP who underwent EMS and have been followed up for more than 5 years.

MATERIALS AND METHODS

Between July 1996 and August 1998, EMS was performed in eight patients with severe common bile duct stenosis associated with CP in our institution. We had experienced 64 patients with CP during that period in the endoscopic retrograde cholangiopancreatography (ERCP) division. The indication for EMS was intractable bile duct stenosis uncontrollable by plastic tube biliary drainage. Ten patients gave their informed consent and the procedure was approved by the Ethical Committee of our institute; however, one patient underwent surgical treatment and another was lost to follow-up. They were all males with a median age of 65.7 years (range: 42-78 years). The etiology of CP was alcohol abuse in seven and idiopathic in one. Underlying diseases were diabetes mellitus in five patients, angina pectoris in one and early colon cancer in one. Mean duration of the illness before manifesting the first symptoms of bile duct stenosis was 3.7 years.

On their first admission, five patients presented with

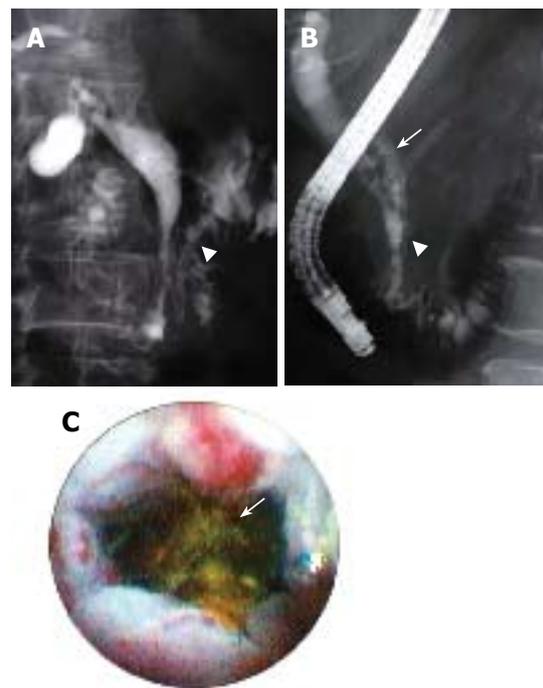


Figure 2 No bile duct stone was observed immediately after EMS in patient number 7 (A), however, stones were noted 6.1 years later (arrow), and they were successfully removed using a basket wire (B). Peroral cholangioscopy revealed the common bile duct distal to the stone (arrow) was patent and the hyperplastic change was not conspicuous (C). Arrow head indicates the pancreatic duct stent.

abdominal pain, six had symptomatic jaundice, and six had cholangitis (Table 1). Serum alkaline phosphatase concentration (ALP) was three times or more the upper limit of normal (mean 1 815 U/L; range 984-3 358) in all the patients. Similarly, serum conjugated bilirubin (D-Bil) was elevated above the upper limit of normal (mean 3.45 mg/dL; range 1.2-7.4) in seven patients. Abdominal ultrasonography and CT scan demonstrated marked dilatation of the common bile duct and slight to moderate dilatation of the intra-hepatic bile duct.

Endoscopic naso-biliary drainage (ENBD) using a 7-Fr. Plastic tube was performed in all the patients as the initial treatment. At that time, cholangiography showed almost complete obstruction or severe stenosis of the lower part of common bile duct. After the resolution of symptoms and laboratory findings of cholestasis by ENBD, three patients were subjected to EMS, while the other five underwent EMS after stenting using a 10 Fr. tube. The mean duration of tube stenting was 1.4 mo (range, 2-7 mo). Three patients developed clogging of the tube stent and subsequently the stent was changed by an EMS. In the other two patients, the tube stents were withdrawn; however, symptoms of re-obstruction were detected within 3 mo and thus EMS was performed.

For EMS, a non-covered, non-self-expandable stent (Strecker stent) was used in two patients and a self-expandable stent (Wallstent, Boston Scientific Corporation, Natick, MA, USA) was used in the other six patients, the length and internal diameter of the stents were 4 and 5 cm, 7 and 10 cm after full expansion, respectively.

In four patients, plastic tube stents were inserted to resolve the main pancreatic duct stenosis, and they were

Table 2 Clinical course of the patients after EMS

| Patient number | Cholestasis | Cholangitis | Stone formation | Dislodgement | Alcohol intake | Outcome | Duration of stent patency(yr) |
|----------------|-------------|-------------|-----------------|--------------|----------------|---------|-------------------------------|
| 1 | + 1 mo | + 1 mo | - | - | - | Dead | 2.8 |
| 2 | + 4.6 yr | - | - | - | - | Alive | 8.3 |
| 3 | + | - | + 7.3 yr | + 5.9 yr | + | Alive | 7.3 |
| 4 | - | - | - | - | - | Alive | 7.1 |
| 5 | - | - | - | + 4.8 yr | - | Alive | 6.9 |
| 6 | - | - | - | - | - | Dead | 5.5 |
| 7 | + | - | + 6.1 yr | - | + | Alive | 6.1 |
| 8 | + 3.6 yr | + 3.6 yr | - | - | + | Dead | 3.6 |

occasionally inserted depending on the patient's symptoms: abdominal and/or back pain.

After EMS, periodical examination of hemogram, blood biochemistry, urinalysis, and abdominal ultrasonography were conducted every 3-4 mo. Examination by cholangiography was occasionally performed, when a patient presented symptoms of biliary obstruction.

RESULTS

Short and medium-term (up to 3 years) follow-up after EMS (Table 2)

EMS was successfully performed in all the patients; however, in one patient (no. 1) clogging was observed within 1 mo and another EMS was inserted inside the first EMS. The patient showed no symptoms of bile duct obstruction thereafter.

The serum concentration of ALP and D-bil markedly decreased after the initial treatment including EMS (Figure 1), and 1 year after EMS, five of eight patients showed normal values, two patients showed slightly higher than the upper limit of normal, and one patient had a high serum concentration of ALP. Two years after EMS, none of the patients showed symptoms of bile duct obstruction and abdominal US demonstrated no dilatation of the intra-hepatic bile duct with the stent located at the original position.

One patient (no. 1) died of acute myocardial infarction 2.8 years after EMS without symptoms of cholestasis during the follow-up period. The course of the other seven patients was uneventful for 3 years.

Long-term follow-up after EMS (Table 2)

One patient (no. 8) died of cholangitis (acute obstructive suppurative cholangitis, AOSC) caused by EMS clogging 3.6 years after EMS. The patient had presented no symptoms up to 1 wk before the last admission, when he felt acute pain in the right upper abdomen and experienced a sudden rise of his body temperature. He was brought to the hospital in a state of shock and was admitted to the ICU, but intensive care including ENBD was not effective and he died 17 d after the last admission. The patient's periodical check up with US and laboratory tests showed no signs of marked cholestasis except slight elevation of serum ALP concentration during the 3.5 years of follow-up as well as 1 mo before. It was suggested from

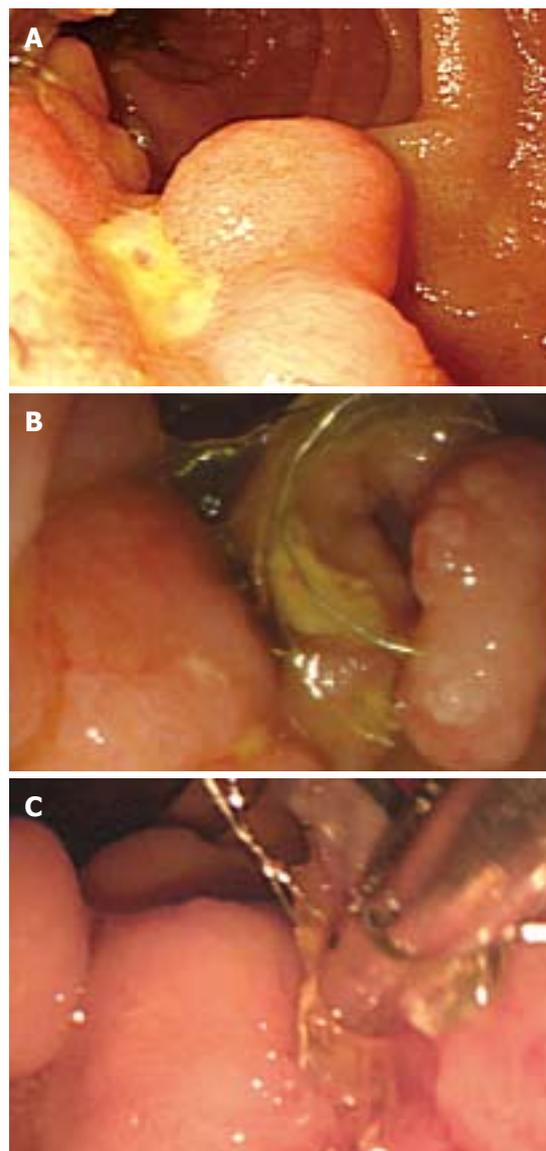


Figure 3 Endoscopic view showing a duodenal ulcer on the side opposite to the duodenal papilla (A) 5.9 years after the EMS in patient number 3. The stent had become dislodged and the steel wires protruded from the papilla (B). They were efficiently cut using the end-cutter (arrow) (C).

the medical chart that the patient had suffered from cholangitis caused by stent clogging 1 wk before his admission, but he did not take it seriously; he thought that the symptoms were those of common cold, and delayed his visit to the hospital. Another patient (no. 2) presented

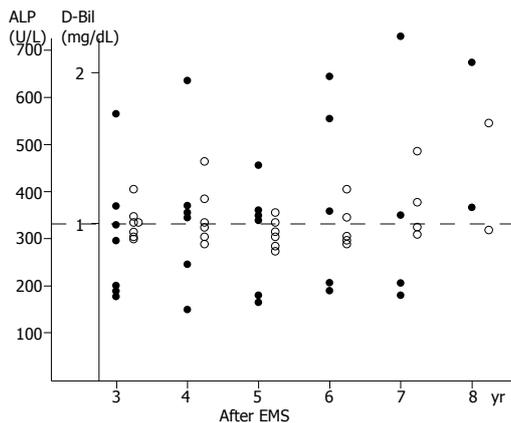


Figure 4 Plotting of the serum alkaline phosphatase concentration (●) and conjugated bilirubin (○) of each patient 3 yr after EMS. The broken line indicates the upper limit of normal.

with signs of cholestasis caused by acute exacerbation of pancreatitis 4.6 years after EMS; however, his condition improved soon after the treatment for pancreatitis without any endoscopic treatment, and he showed no symptoms thereafter. Two other patients (nos. 3 and 7) presented with cholestasis caused by choledocholithiasis in the upper part of the CBD proximal to the stent 7.3 and 6.1 years after EMS, respectively. In one patient (no. 7) the stones were successfully removed with a basket wire and he presented no symptoms thereafter (Figures 2A and 2B). In the other patient (no. 3) the stone could not be removed with a basket wire and two sessions of endoscopic hydraulic lithotripsy were necessary to eliminate it. Cholangioscopic examination of these patients showed that the metallic mesh was embedded into the bile duct wall allowing sufficient inner space without epithelial hyperplasia within the stent (Figure 2C).

Two patients (nos. 3 and 5) suffered from duodenal ulcers at 5.9 and 4.8 years after EMS, respectively, because the EMS wire dislodged and protruded from the papillary orifice, contacting the duodenal wall on the opposite side. The wire of the protruding part was cut under endoscopy with the end-cutter (Olympus, Tokyo, Japan), and the ulcer healed later (Figure 3).

Patient (no. 6) died of maxillary carcinoma 5.5 years after EMS although he presented no symptoms of cholestasis during the follow-up period.

Thus, five of the eight patients are alive at present and their mean follow-up period is already 7.4 (range: 6.5–8.3 years) years. The value of ALP is over the upper limit of normal in three patients now (Figure 4), and mild dilatation of the intra-hepatic bile duct is observed in three of them.

Patients (nos. 3, 6, 7, and 8) continued drinking alcohol after EMS; two of them developed choledocholithiasis and one suffered from AOSC. The other four patients did not drink alcohol after EMS and only one showed mild, transient signs of cholestasis.

DISCUSSION

EMS for benign bile duct stenosis as a complication of CP has been attempted in some institutes and patency of the stent is likely to be better^[12–16]. Kahl *et al* used metal stents

as the permanent treatment option and their patients remained free of obstructive jaundice or cholangitis for 1 year^[16]. Deviere *et al* conducted a prospective study with a mean follow-up of 33 mo, and the stent lumen remained patent and functional throughout the follow-up period^[12]. The reported results agree with our study concerning short to medium-term follow-up after EMS. In fact within 3 years after the treatment, all the patients except one who died of a heart attack showed almost uneventful clinical courses regarding biliary stenosis; judging from symptoms, laboratory findings including ALP, D-Bil, and imaging findings. One elderly patient died of causes other than CP with no signs of bile duct re-obstruction for 2.8 years. Such a good outcome was not achieved with the plastic tube stent^[5,6,8,9,17], and long-term outcome of treatment with multiple tube stents was also disappointing^[18].

Based on these results, EMS is obviously better than tube stenting regarding patency and not requiring scheduled changing of the stent. These advantages are thought to come from the characteristics of EMS: large caliber and expanding effect. However, the main problem of EMS is the development of epithelial hyperplasia in the stent resulting in obstruction^[18]. Deviere *et al* reported that only two of 20 patients developed epithelial hyperplasia leading to cholestasis and jaundice within six months after stenting^[12]. One of our patients also developed early obstruction of EMS, but an additional stent resolved this difficult problem. The metallic mesh is thought to become embedded in the bile duct wall early after stenting, so that a continuous membrane covers the inner stent^[12]. Based on this hypothesis, ingrowth within the stent and subsequent obstruction may be related to the tumor rather than to CP, and in fact, the majority of our patients did not develop an early obstruction.

Although good results have been reported after a short- to medium-term follow-up, long-term results are unknown. Hastier *et al* reported the outcome of a patient treated with a metallic stent, who presented with no evidence of recurrence of cholestasis or episodes of cholangitis for a relatively long period of 3 years^[14]. Van Berkel *et al* recently reported that they treated 13 patients with biliary stricture due to CP by self-expanding metal stent (SEMS) with the mean follow-up for 50 mo and that SEMS was found to be safe and provides successful as well as prolonged drainage in selected patients^[19]. Nevertheless, the opinions in our institute were controversial regarding uncertainty of safety and outcome of EMS in patients with CP after a long-term follow-up, and we voluntarily decided to stop the application of EMS treatment from August 1998 until December 2003, when we could assess the long-term outcome and re-started EMS. Since then, two patients have been subjected to EMS up to now.

The outcome of EMS after more than 3 years was fairly good. But we experienced a very serious complication caused by re-obstruction of the bile duct. Emergency treatment was delayed leading to disappointing results in this case. If adequate intensive treatment had been started much earlier, the patient would have been saved. Another patient showed mild cholestatic symptom due to acute exacerbation of pancreatitis, but recovered soon after pancreatitis resolved without any intervention,

suggesting cholestasis was not caused by clogging of the stent but by another factor such as papillary edema. In two other patients with stone formation, cholangioscopic findings showed the patency of the stent lumen, according to the assumption of Deviere *et al*^[12]. However, stones were thought to develop as a consequence of stagnation of bile, accordingly complete bile flow may be difficult to obtain even with EMS. Another complication of dislodgement was duodenal ulcers which were well controlled by endoscopic treatment.

One of our patients died of maxillary cancer long after EMS. Hastier *et al* decided to insert a metallic stent in their patient in view of his poor prognosis associated with the pulmonary malignancy^[14]. CP is shown to frequently associate with various malignancies and a significantly lower survival rate compared with non-CP^[20-22]. Therefore, in choosing EMS, this is one of the decision-making facts as well as the patients' general conditions and the lack of response to stenting using a plastic tube^[2,13].

It is difficult to determine the optimal indications for EMS from our results because of the limited number of patients and the absence of a control group. However, since five of the eight patients are alive and leaving ordinary lives 7.4 years after EMS, this procedure is evidently one of the very promising options of treatment for bile duct stenosis in patients with CP, provided they are closely followed up through periodical check-ups. Thus setting a system for prompt management in case of an emergency is desirable. In addition, our results indicate that alcohol intake may be related to the poorer prognosis of patients subjected to EMS; thus prohibition of alcohol consumption is essential.

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Favorable outcomes of hilar duct oriented hepatic resection for high grade Tsunoda type hepatolithiasis

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Abstract

AIM: To evaluate the efficacy of hilar duct oriented hepatectomy for intractable hepatolithiasis, the ventral hilum exposure (VHE) method that has been applied by the authors.

METHODS: From June 1994 to June 2004 for a period of 10 years, 153 patients who had Tsunoda type III or IV hepatolithiasis, received hepatectomy at our institution. Among these patients, 128 who underwent hepatectomy by the VHE method were the subjects for the study. We analyzed the risk of this procedure, residual rate of intra-hepatic stones, and stone recurrent rates.

RESULTS: The average age was 54.2 years, and the male to female ratio was 1:1.7. The average follow-up period was 25.6 mo (6-114 mo). There was no post-operative severe complication or mortality after the operation. The rate of residual stones was 5.4% and the rate of recurrent stones was 4.2%.

CONCLUSION: VHE is a safe surgical procedure and provides favorable treatment results of intractable hepatolithiasis. Especially, this procedure has advantage in that intra-hepatic bile duct stricture may be confirmed and corrected directly during surgery.

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Key words: Hepatolithiasis; Hepatic resection; Residual stone; Recurrent stone

Kim BW, Wang HJ, Kim WH, Kim MW. Favorable outcomes of hilar duct oriented hepatic resection for high grade Tsunoda type hepatolithiasis. *World J Gastroenterol* 2006; 12(3): 431-436

INTRODUCTION

Although hepatolithiasis is a benign condition, the clinical progression of this disease not only often limits a patient's social activities, sometimes to an extreme degree, but also may lead to liver parenchymal destruction due to recurrent cholangitis or even septic death if adequate treatment is not provided. Moreover, cholangiocarcinoma is found in approximately 5-10% of patients with hepatolithiasis^[1,2]. The incidence of hepatolithiasis in the Korean population has been reported as 15% of all biliary tract stones, which is relatively higher than the data reported in Western populations^[3].

In clinical practice, however, the treatment results are less favorable when compared to those of extrahepatic bile duct stones. The reason for this is the stricture of the intra-hepatic duct as a major cause of hepatolithiasis^[4,5], which is commonly intractable because of the anatomical characteristics. It has been known that the most important aspects in the treatment of hepatolithiasis is the complete removal of all intra-hepatic stones and resolution of accompanied intra-hepatic duct stricture, in order to reduce the incidence of post-operative cholangitis and stone recurrence^[6].

Recent developments in endoscopic techniques have also been shown to result in favorable outcomes^[7]. Such advances have led to the shifting of surgical practice from uniform surgery to a combination of surgery and endoscopy for the treatment of hepatolithiasis^[8,9]. But the endoscopic approach for hepatolithiasis is not always successful due to various intra-hepatic duct anatomies and cannot solve concomitant liver atrophy or latent cholangiocarcinoma.

The authors of this study implemented a surgical approach for hepatolithiasis according to the Tsunoda classification of hepatolithiasis, which describes the intra-hepatic duct in terms of dilatation and strictures, and is as follows: type I, no marked dilatation or strictures of intra-hepatic bile ducts; type II, diffuse dilatation of the intra-hepatic biliary tree without intra-hepatic duct strictures and frequently a stricture of the distal common bile duct; type III, unilateral solitary or multiple cystic intra-hepatic dilatation, frequently accompanied by stenosis of the left or right intra-hepatic bile ducts; and type IV, the same attributes as type III but with bilateral involvement of hepatic lobes^[10]. The authors' policy of treatments in hepatolithiasis is according to Tsunoda type, that is, the type I or II is treated with endoscopic approach and the type III or IV is with surgical approach.

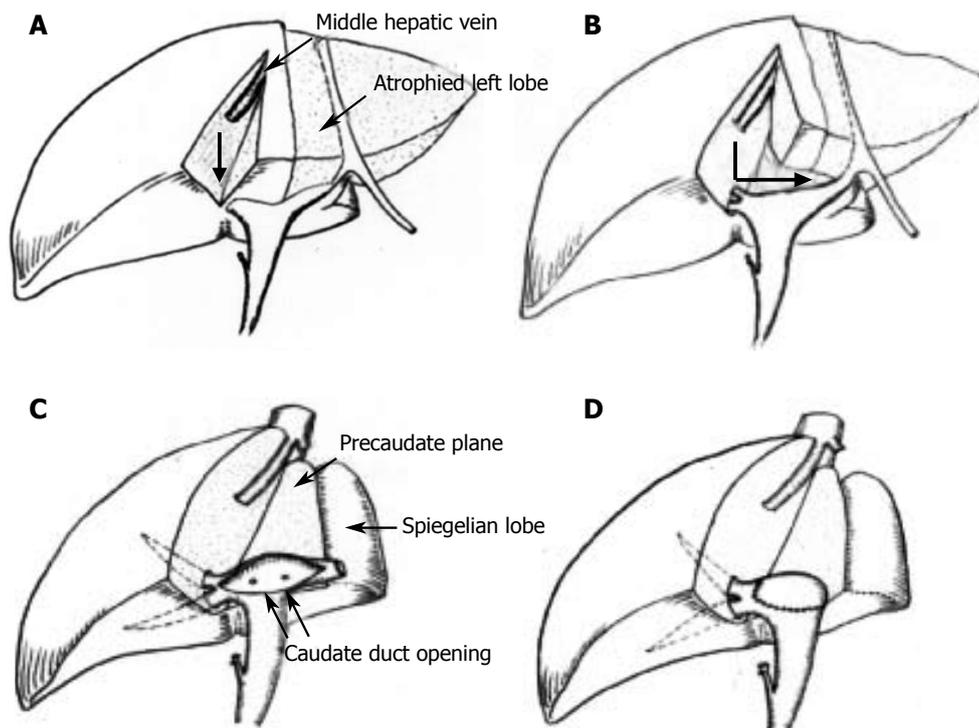


Figure 1 Schematic drawing of VHE procedure during left lobectomy. **A:** Hepatotomy commences vertically from the Cantlie's line to the direction of the hilar bile duct (thick arrow); **B:** after reaching the hilum, the hepatotomy is continued along the pre-caudate plane (angled thick arrow); **C:** the ventral portion of the hilar hepatic duct is opened along its direction; **D:** after completion of the necessary ductal procedure.

MATERIALS AND METHODS

From June 1994 to June 2004, a retrospective review was undertaken of 153 patients who received partial hepatectomy for hepatolithiasis at our institution, among whom, 128 patients underwent hilar duct oriented hepatectomy (ventral hilum exposure method, VHE). We analyzed the surgical risk of VHE method, residual rate of intra-hepatic stones, stone recurrent rates, and those patients with concomitant cholangiocarcinoma were also investigated.

The definition of hepatolithiasis was according to the description by Couinaud in which a gallstone is present in the proximal portion of the confluence of the common hepatic duct. The presence of residual or recurrent hepatolithiasis after surgery were assessed by ultrasonography (US), computerized tomography (CT) scans, cholangiograms by endoscopic retrograde cholangiopancreatography (ERCP) and/or percutaneous transhepatic cholangiography (PTC). The postoperative follow-up comprised of US every 3 mo and CT scan every 6 mo for the first three years, and then once a year thereafter, or when abnormal symptoms suspicious of cholangitis or residual or recurrent hepatolithiasis were present. If the residual or recurrent stones were suspected by US or CT scan, the PTC and/or ERCP were introduced.

Recurrent hepatolithiasis was defined as stones recurring after 2 years of initial therapy. Operative mortality and hospital mortality was defined as death of a patient within 30 d of surgery, and death before discharge from the hospital after surgery, respectively.

Operative techniques

The goal of VHE is total exposure of the hilar bile duct. In case of left lobectomy, first the course of middle hepatic vein

and the distribution of intra-hepatic stones are confirmed by intra-operative US, then the gallbladder is removed. A sagittal liver parenchyma dissection (hepatotomy) along the Cantlie's line or midline of the GB bed is done until reaching the right hilar Glisson's sheath. Then the sagittal dissection plane is rotated 90° to the left along the coronal plane or pre-caudate plane^[1]. The preservation of the middle hepatic vein during sagittal hepatotomy is optional. Managed thus, long segment of the ventral portion of hilar Glisson's sheath can be separated from dorsal part of the segment IVb liver parenchyma (Figures 1A and 1B). Without exception in our experience, the hepatic hilar bile duct is situated at the extreme antero-superior aspect in the hilar Glisson's pedicle that can be confirmed by needle puncture aspiration of bile, and then the anterior wall of the hilar bile duct can be opened safely along its direction without the need of dissection of hilar Glisson's sheath (Figures 1C and 1D).

In this way, direct visualization of the openings of the second order branches of the intra-hepatic bile duct allows pronounced accuracy of intra-operative cholangioscopy in each of the intra-hepatic duct branches, and precise evaluation of the distribution of intra-hepatic stones and hilar stricture, and plastic reconstruction of the strictured hilar hepatic duct. Plastic reconstruction of intra-hepatic duct results in avoidance of a hepatico-jejunostomy in almost every patient, and also primary closure of the bile duct incision site maintains the physiologic anatomy of the intra-hepatic bile duct. The VHE procedure also resulted in easier confirmation of the common bile duct status, and none of the patients who received this therapy required a T-tube choledochostomy (Figures 2 and 3).

We did not perform VHE in cases where the hepatolithiasis was situated in the third branch or the more periphery of the intra-hepatic bile duct, when parenchymal destruction of the liver was observed in the periphery, and when no stricture was present up to the second

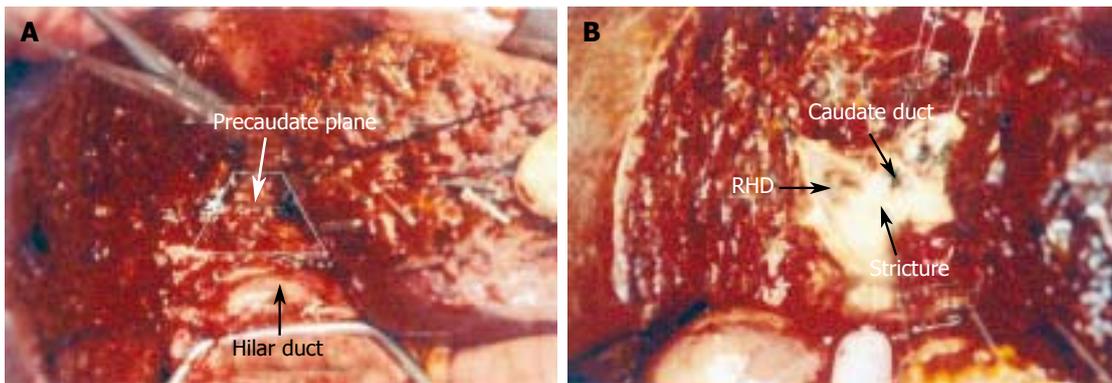


Figure 2 A: Dissecting along the pre-caudate plane allows the ventral portion of the hilar duct to be exposed during left lobectomy; **B:** the hilar duct was opened along its direction; RHD: right hepatic duct opening.

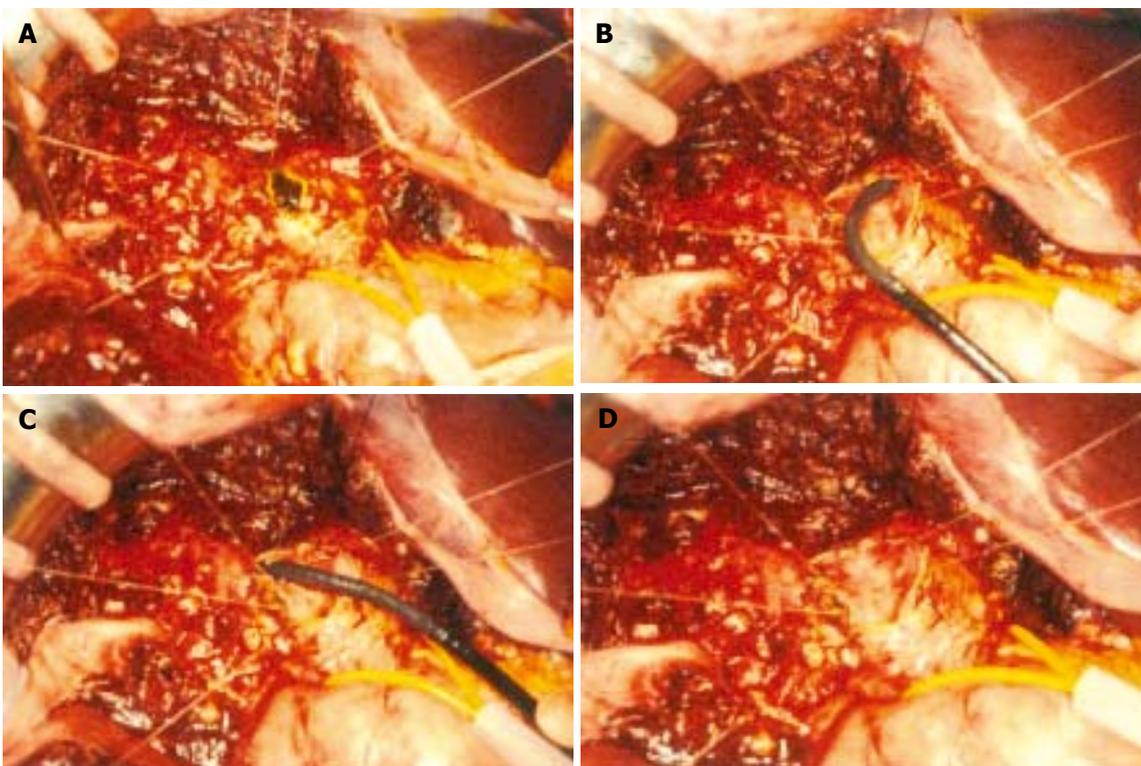


Figure 3 During central lobectomy, the hilar duct was exposed and opened. **A:** Extraction of the IHD stones; **B and C:** application of intra-operative cholangioscope to the left and right IHD; **D:** after the removal of all intra-hepatic stones.

order branches of the hilar bile duct. These patients were managed with conventional liver parenchyma resection (Table 1).

RESULTS

The average age of patients who received VHE surgery was 54.2 years, and the male to female ratio was 1:1.7. All 128 patients who underwent VHE surgery were Tsunoda type III or IV (98 patients were Tsunoda type III, and 30 were type IV). The type of stone in all the 153 patients who received surgery was pigment stone, and hilar duct stricture was confirmed in 90 of 128 patients who had VHE surgery (70%). In the remaining 38 patients without hilar ductal stricture, strictures were observed in the

second order branches.

The average follow-up period of 128 patients after VHE for hepatolithiasis was 25.6 months (range; 6-114 mo). The number of patients with residual stones after VHE hepatectomy was 7/128 (5.4%), and residual stones were found in 4/98 patients with Tsunoda type III (4.1%) and in 3/30 with type IV (10%). Among them, there were six patients in whom residual stone removal by PTCS was performed who had symptoms such as cholangitis. And the remaining four asymptomatic patients were being followed up only with observation to date.

We could follow-up 48 patients with CT scan and US for more than two years after complete stone removal by the VHE method. The range of the follow-up period of these 48 patients was 26-116 mo, with an average of

Table 1 Type of hepatic resection

| Type of operation | n | |
|------------------------------------|-----|--------------|
| Explo-laparotomy only ¹ | 6 | Non-VHE (25) |
| LLS ² | 14 | |
| LLS+RPS ³ | 2 | |
| RPS | 3 | |
| CL ⁴ | 4 | VHE (128) |
| LL ⁵ ±subsegmentectomy | 114 | |
| RL+IVb ⁶ | 10 | |
| Total | 153 | |

¹Explo-laparotomy only due to carcinomatosis of cholangiocarcinoma. ²Left lateral segmentectomy. ³Right posterior segmentectomy. ⁴Central lobectomy. ⁵Left lobectomy. ⁶Right lobectomy+subsegmentectomy IVb.

Table 2 Rate of residual and recurrent stones after VHE procedure (n = 128) according to Tsunoda type

| Tsunoda type | Number of residual stone (%) | Number of recurrent stone (%) |
|--------------|------------------------------|-------------------------------|
| III | 4/98 (4.1) | 2/39 (5.1) |
| IV | 3/30 (10) | 0/9 (0) |
| Total | 7/128 (5.4) | 2/48 (4.2) |

60.4 mo. Among them, only two patients had recurrent stones, showing a recurrence rate of 4.2% (2/48). Both of these patients were Tsunoda type III at the time of surgery (Table 2). And these two recurrent patients developed cholangitis, and one died of sepsis as a result of the cholangitis. Among the 46 patients who were followed up for more than 2 years without recurrent stones, 2 developed acalculous cholangitis as the complication and were managed by conservative treatment. One patient was diagnosed with cholangiocarcinoma 3 years after surgery without evidence of stone recurrence (Table 3).

Among the total of 153 patients who received hepatectomy for hepatolithiasis, there were 17 cases of cholangiocarcinoma (11%), and six of these patients were of advanced state of unresectable disease during laparotomy. There were also three patients with *in situ* cholangiocarcinoma after confirmation of pathology. And only three patients were pre-operatively determined to be with cholangio-carcinoma among the total of 17 cases of concomitant cholangiocarcinoma.

There was no case of operative mortality or hospital mortality in all patients who were studied. Post-VHE complications occurred in 33/128 patients (25.7%), and consisted of wound infections (20 patients, the most common complication), pulmonary complications such as pleural effusion or pneumonia (nine patients), bile leakage (four patients), and one case each of post-operative minor intra-abdominal hemorrhage, intraperitoneal abscess, and gastrointestinal bleeding. All of the complicated cases were successfully managed conservatively.

DISCUSSION

Recent therapy by the combination of endoscopy and

Table 3 Long-term results in patients (n = 48) who underwent complete stone removal by VHE

| Long-term results | Number of cases (%) |
|--|---------------------|
| Free of symptoms and no recurrent stones | 43 (89.6) |
| Recurrent stones with cholangitis | 2 (4.2) |
| Cholangitis without recurrent stones | 2 (4.2) |
| Cholangiocarcinoma | 1 (2.1) |

surgery for the treatment of hepatolithiasis has allowed overcoming the limitations of each modality and therefore has led to marked enhancement of treatment results. Another reason for the increased treatment success may be due to the more accurate pre-operative evaluation of intra-hepatic bile duct strictures and distribution of intra-hepatic stones.

Many favorable results have been reported in the literature with regard to endoscopy in the management of hepatolithiasis^[7,12]. In our institutional experience, analysis of endoscopic treatment results of 106 patients with hepatolithiasis has shown that the overall residual stone rate was 16%, but this rate was higher in patients with Tsunoda types III and IV (24% and 30%, respectively). We thought that this result was from the intra-hepatic stricture and the sharp angled branches of the intra-hepatic bile duct anatomy^[13,14]. And it is well known that there are major limitations of endoscopic hepatolithiasis, such as parenchymal destruction of the liver, liver abscess formation, and a 5-10% incidence of cholangiocarcinoma that occurs in patients with hepatolithiasis^[1,2,15,16]. Our study revealed a slightly higher rate (11%) of patients found with cholangiocarcinoma, compared to previous reports from other institutions, and the reason for this is thought to be due to the fact that patients who received surgery in our institution were those with parenchymal destruction and intra-hepatic bile duct stricture, and also because a larger number of patients were with long-standing, advanced hepatolithiasis. Another serious problem in patients with hepatolithiasis accompanied by the presence of cholangiocarcinoma is that the latter disease is not easily detected in the pre-operative evaluation, and thus may be missed during endoscopic treatment of hepatolithiasis^[2,17]. This was also our finding in this study.

Endoscopic treatment of hepatolithiasis is considered to be relatively safe^[18]. However, to endoscopically remove all stones completely and to resolve stricture of the intra-hepatic bile duct, the procedure is conducted on an average of 3 to 4 times at 1-2 wk intervals, restricting the patients' activities over a long period of time, even though the procedure entails less physical and psychological burden for the patient than surgical operation.

In Korea as well as in most Asian populations, pigment stones are the most common, while cholesterol stones are predominant in Western populations. This was also the case in our study in which all patients who received surgery were with pigment stones. In contrast to cholesterol stones, pigment stones demonstrate different clinicopathologic features of the intra-hepatic bile duct. Most cholesterol hepatolithiasis are usually located in the

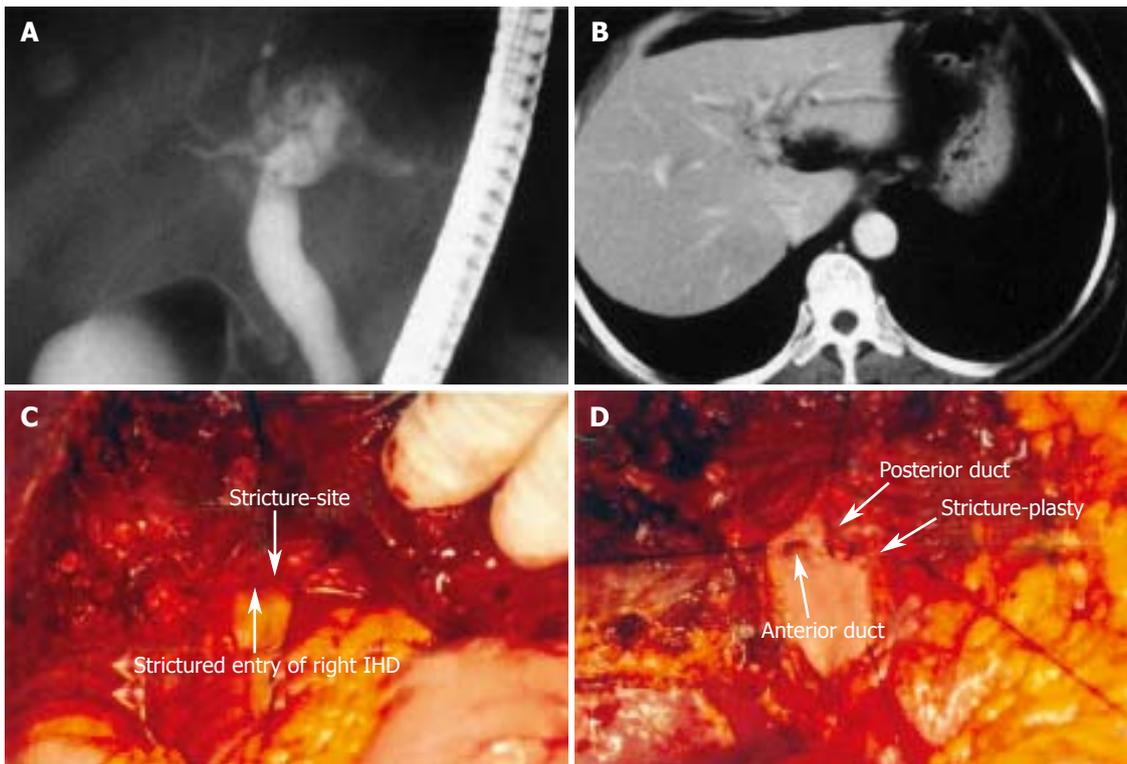


Figure 4 A and B: Pre-operative imaging of hepatolithiasis with hilar duct stricture; C: opened hilar IHD shows narrow entry of right IHD (arrow); D: after stricture-plasty both entries of the right anterior and posterior ducts are seen.

peripheral intra-hepatic ducts, accompanied by a lesser incidence of cholangitis, there is less wall thickening of the stone-containing duct, and the presenting symptoms are usually mild. However, pigment stones are frequently accompanied by hilar duct stricture and hilar duct stones, show severe wall thickening of the stone-bearing duct, and the symptoms are more severe due to a higher incidence of cholangitis^[19]. According to our knowledge of previously reported literature, we have not been able to find any reports with regard to the rate of hilar duct stricture in hepatolithiasis patients due to pigment stone. We, however, were able to observe in our study that hilar stricture was present in 90 of 153 (59%) patients. Therefore, it is the opinion of the authors that during surgery for pigment stone hepatolithiasis, resection of the stone bearing liver parenchyma and removal of stones only is inadequate because the possibly accompanied hilar strictures will make recurrent diseases, and which must be appropriately corrected during surgery.

The VHE procedure adopted and analyzed by the authors of this study has been established as a safe method in a previous report of the pre-caudate plane of the paracaval portion of the caudate lobe^[11]. Anatomically, the pre-caudate plane may be described as a plane bounded inferiorly by the hilar Glisson's pedicle, superiorly by the dorsal portion of origin of the middle hepatic vein, the left side by the ligamentum venosum, and the right side by the imaginary line of the second order branching point of the right Glisson's pedicle and the dorsal portion of origin of the right hepatic vein^[11,20].

According to the experience of the authors of this study, we did not observe any postoperative incidence

of severe hemorrhage of the resection plane or hypoxic damage of the caudate lobe during hepatotomy along the pre-caudate plane for left lobectomy or extended right lobectomy of the liver.

This VHE method also permitted facilitated understanding of individual hilar bile duct anatomy, the openings of second order branches from the hilar bile duct by completely exposing the ventral hilar Glisson's pedicle and then incising along the hilar bile duct. It also allowed increased accuracy of the intra-operative choledochoscopy, complete removal of intra-hepatic stones without difficulty under direct visualization, and easier stricture-plasty of the constricted hilar bile duct.

Our experience showed that the opening of hilar bile duct along its direction will allow visualization of 3 or 4 openings of the second order branches of the intra-hepatic bile duct, which emerge beginning from the right side in the following order; the anterior segmental branch, the posterior segmental branch (or common duct opening of right anterior and posterior hepatic duct), and 2 caudate branches. In many instances, the order and the number of caudate branches varied from patient to patient, and the order frequently presented as anterior segmental branch, caudate branch, posterior branch, and then the caudate branch. When hilar bile duct stricture is present, the stricture site may exist anywhere in the openings of the second order branches, thus necessitating adequate exposure of the hilar bile duct for direct visualization of the inside of the hilar bile duct for preservation of each second order branches during stricture-plasty (Figure 4).

Whenever possible, the authors did not perform a bilioenterostomy after hepatectomy but attempted to

conserve the anatomy of the bile duct physiologically. This was because according to two studies by Kusano *et al*^[21] and Jan *et al*^[22], incidence of cholangitis was high after bilioenterostomy.

In this study, the 5.4% rate of residual stones and 4.2% of recurrent stones after VHE surgery for hepatolithiasis were much better results than historical control datas of conventional hepatectomy that showed residual stone rate about 10% and recurrent stone rate about 12-29.6%^[22-25], and this may be attributed to the high efficacy of the VHE procedure in correcting the bile duct stricture. Moreover, as the patients who underwent VHE for hepatolithiasis in this study were all high grade Tsunoda type which is difficult to remove by endoscopic methods, the effectiveness of the proposed VHE method is further stressed. We also emphasize the complication rate of VHE which was comparable with that of conventional hepatectomy.

In conclusion, we present in this study the VHE method that allows accurate assessment of the pathologic anatomy of the intra-hepatic bile duct during hepatectomy for hepatolithiasis. We also suggest from our observed data that this procedure will decrease the residual stone rate and long term recurrent stone rate after surgery without serious complications. We recommend this procedure for surgical treatment of intractable hepatolithiasis.

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Differential c-erbB-1 and c-erbB-2 mRNA expression in cancer of the pancreas compared with cancer of the papilla of Vater

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Abstract

AIM: We examined quantitative mRNA expression of growth factor receptors (c-erbB-1, c-erbB-2) and the anti-apoptosis gene survivin known to be regulated in pancreatic adenocarcinomas and compared the expression pattern with that in carcinomas of the papilla of Vater.

METHODS: Quantitative real-time reverse transcriptase-PCR (QRT-PCR, Taqman™) was performed to analyze mRNA expression levels of c-erbB-1, c-erbB-2 and survivin in normal and corresponding tumor samples of 31 pancreatic adenocarcinomas and 8 cancers of the papilla of Vater.

RESULTS: The overall median mRNA expression of survivin was significantly increased in both adenocarcinoma of the pancreas ($P < 0.01$) and papilla of Vater ($P < 0.008$) compared with uninvolved normal control tissue. In pancreatic cancer, expression of c-erbB-1 was significantly decreased compared with the normal pancreatic tissue ($P < 0.03$), whereas in the cancer of the papilla of Vater expression of c-erbB-2 was significantly downregulated ($P < 0.05$) compared with the paired normal samples. Gene expression was not associated with tumor stage, differentiation or prognosis.

CONCLUSION: The common anti-apoptosis gene survivin is overexpressed both in the cancer of the papilla of Vater and pancreas. In contrast, the growth factor receptor genes c-erbB-1 and c-erbB-2 are differentially regulated in both tumor entities adding further evidence that pancreatic cancer is biologically different from the cancer of papilla of Vater.

INTRODUCTION

Adenocarcinoma of the pancreas has the worst prognosis of all cancers, with a 5-year survival rate less than 3%, accounting for the fifth highest number of cancer related deaths in North America and Europe with a rising incidence^[1-3]. The only curative treatment for pancreatic cancer (PC) is complete resection which is possible in only 10-20% of patients^[4] and associated with relatively constant 5-year survival rates of approximately 20% for the last 30 years^[5,6,7]. The dismal prognosis and the lack of effective therapeutic regimens for this disease are attributed to several causes: (a) PC shows an aggressive biological phenotype, which is characterized by the early invasion of surrounding structures and rapid metastatic spread^[8,9]; (b) radiation therapy and chemotherapy are reported to be effective only in selected patients after tumor resection^[9-11]; (c) although various genetic alterations were identified, which have improved our understanding of the carcinogenesis of this disease, the responsible molecular mechanisms are largely unknown. New treatment regimens based on molecular classifications of the individual tumor may improve the outcome for patients with PC.

In contrast, cancer of the papilla of Vater (CPV) has a better prognosis with a 5-year survival rate of over 40% after curative resection^[12,13]. CPV is a rare disease representing 6-12% of all periampullary malignancies^[14] with an estimated incidence of 2.9 per million^[15]. Because of its anatomical location, CPV becomes symptomatic at an earlier stage than PC. Therefore, the majority of the patients with CPV are candidates for surgical therapy. Evidence is increasing that differences in the tumor

biology of these two entities contribute to the different prognosis^[16].

Survivin is a member of the inhibitor of apoptosis gene family, which is unique for its expression in a wide range of embryonic and fetal tissues, whereas almost no transcripts are detected in terminally differentiated normal adult tissues. In human pancreatic islets survivin expression is also developmentally regulated^[17]. Survivin expression in human fetal islets was identified immunohistochemically in alpha and beta islet cells whereas adult pancreases showed a staining only in the alpha cells. However, it is re-expressed in several human cancers^[18] and has an additional function in the regulation of cellular proliferation^[19] and angiogenesis^[20] of cancers. Survivin expression has been reported to be associated with poor survival in patients with colorectal cancer^[21,22] and several other human cancers^[23]. In a recent immunohistochemical study on 52 PC patients, increased survivin expression was strongly correlated to a higher proliferative index, nevertheless no correlation between survivin expression and survival of the patients was shown^[24].

The epidermal growth factor receptor (EGF-R) family contains four structurally homologous transmembrane proteins with intracellular tyrosine kinase activity. The best-described growth factor receptors of this family are EGF-R also known as c-erbB-1^[25] and the c-erbBs-2, 3, and 4^[26]. They share a significant sequence homology and are frequently overexpressed in PC. Activation of the receptors leads to increased DNA synthesis, and changes in cell motility and cell metabolism^[27]. EGF-R is activated by a family of peptide ligands that includes EGF, TGF- α , heparin binding EGF-like growth factor, betacellulin, and amphiregulin^[28]. The EGF-R is encoded by the c-erbB-1 proto-oncogene and is a transmembrane growth factor with tyrosin kinase activity. In normal pancreas, c-erbB-1 is expressed only in the islets of Langerhans. Nevertheless the c-erbB-1 gene is overexpressed in human pancreatic cell lines and in 95% of ductal adenocarcinomas, due to an increase in gene transcription^[29]. Overexpression of c-erbB-1 detected by immunohistochemistry is correlated with reduced survival rates in several studies^[30,31].

The c-erbB-2 (also called HER2/neu) gene encodes a 185-ku transmembrane glycoprotein with tyrosine kinase activity, and acts as a receptor for a class of ligands that includes the heregulins, gp30, and NEU-differentiation factor. C-erbB-2 is overexpressed in the bladder, breast, esophageal, and gastric cancer, where it appears to have a role in lymphatic tumor spread^[32,33]. In ductal adenocarcinomas of the pancreas and in ampullary tumors, c-erbB-2 is overexpressed in 20%, which is usually caused by gene amplification^[34]. Reports about the prognostic significance of c-erbB-2 are not uniform throughout the literature. One study showed a survival advantage for patients without the overexpression of c-erbB-2 in their tumors^[35], while another study showed a correlation of c-erbB-2 serum levels and survival of the patients^[36]. In the majority of studies no prognostic significance for the expression of c-erbB-2 in PC was revealed^[37,38].

In the present study we have determined gene expression levels of survivin, c-erbB-1 and c-erbB-2 in PC and CPV applying quantitative real-time RT-PCR (TaqMan

TM) in order to look for gene profiles that might account for the different biological behavior.

MATERIALS AND METHODS

Patients and specimens

Between July 1997 and December 2003 fresh frozen tumor and corresponding normal tissue from 63 patients, who underwent curative resection of suspected or proven pancreatic or ampullary tumors were collected. Informed consent was obtained from all the patients. Data and tissue collection were in accordance with the regulation of the local ethic committee. Tissues were frozen immediately in liquid nitrogen and stored at -80 °C until further analysis.

The definitive histology of the tissue used for RNA isolation was confirmed in serial sections by a staff pathologist. Only tumor specimens with at least 75% malignant cells were used for RNA isolation.

Finally, 31 specimens from ductal adenocarcinomas of the pancreas and 8 from adenocarcinomas of the papilla of Vater with paired normal control tissues were available for gene expression analysis. This study population consisted of 24 (62%) men and 15 (38%) women with a median age of 59.4 years (range, 33-81 years). Tumor staging was performed according to the International Union Against Cancer (UICC) tumor-node-metastasis classification. In patients with PC, 1 (3.2%) patient had a stage I tumor, 3 (9.6%) patients had a stage II tumor, 24 (77.4%) patients had a stage III, and 3 (9.6%) had a stage IVa tumor. In patients with CVP, 5 patients presented with a stage II tumor, 2 patients had stage III, and 1 patient a stage IV tumor.

Twenty four patients underwent a Kausch-Whipple procedure, and in 10 patients the pylorus was preserved. In four patients a left-sided pancreatic resection and in one patient a pancreatectomy was performed. In patients with cancer of the papilla ($n=8$) of Vater, five received a pylorus preserving partial pancreaticoduodenectomy, whereas three patients underwent a Kausch-Whipple procedure. The median follow-up for surviving patients was 9.5 mo and no patient was lost to follow-up.

RNA isolation

Total cellular RNA was isolated using RNeasy Mini Kit (Qiagen, Hilden) adding proteinase K (0.2 mg/mL) and quantified at $A_{260/280\text{ nm}}$ (SmartSpec; Bio-Rad, Hercules, CA, USA).

Quantitative RT-PCR

Total cellular RNA (0.5 μg) was reverse-transcribed as described previously^[39]. An amount of 25 ng of cDNA was taken for real-time PCR using the TaqMan ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Darmstadt, Germany). To normalize the amount of total RNA present in each reaction, the housekeeping gene β -actin was amplified.

Primers and probes were designed to encompass intron between exon sequences using the Primer Express Software (Applied Biosystems, Darmstadt, Germany). The β -actin probe was labeled with 5'-VIC and 3'-Minor Groove Binder/Non-Fluorescent Quencher (Applied

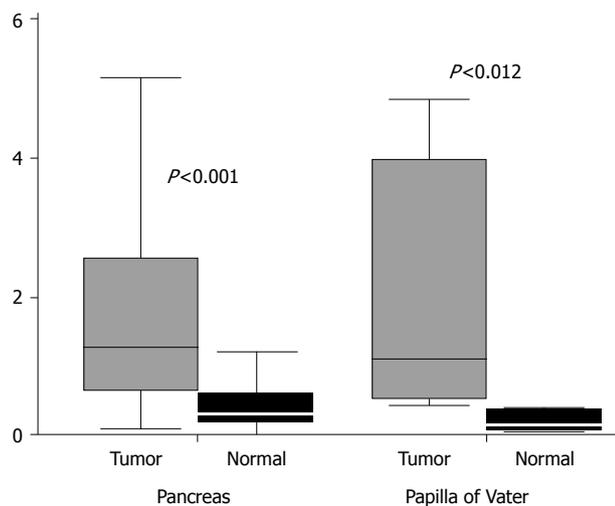


Figure 1 Expression of survivin in PC ($n=31$) and CPV ($n=8$).

Biosystems, Darmstadt, Germany). The survivin, c-erbB-1 and c-erbB-2 probes were labeled at the 5' end with FAM and at the 3' end with the quencher TAMRA (Eurogentec, Seraing, Belgium). The sequences for the primers and probes were as follows: survivin described by Warnecke-Eberz *et al* 2005. C-erbB-2 sense 5' CCA GGA CCT GCT GAA CTG GT 3', anti-sense 5' TGT ACG AGC CGC ACA TCC 3', probe 5' CAG TTG CCA AGG GGA TGA GCT ACC TG 3'. C-erbB-1 sense 5' CGC AAG TGT AAG AGT GCG AA 3', anti-sense 5' CGT AGC ATT TAT GGA GAG TGA GTC T 3', probe 5' CCT TGC CGC AAA GTG TGT AAC GGA AT 3'. The reliability of PCR amplification and detection was verified on serial dilutions of standard cDNAs prior to analyses of patient samples. To ensure that no genomic DNA was amplified the assays were checked with RNA samples minus reverse transcription control as well as with genomic DNA as template.

The PCR reaction was performed as described previously^[39]. All analyses were done in triplicates. Gene expression levels were calculated using standard curves generated by serial dilutions of placenta cDNA (Clontech Laboratories, Palo Alto, CA, USA).

Statistical analysis

The gene expression analyses yielded values, which were expressed as ratios between two absolute measurements: the gene of interest and the internal reference gene β -actin. Gene expression levels were described using the median as point estimator and the range of values. Cut-off values for discrimination of dichotomized mRNA expression levels and clinicopathologic parameters were derived from receiver operating curve data (area under the curve and the 95% confidence interval) according to Metz *et al*^[40]. Associations between gene expression levels and clinicopathological parameters were evaluated using the χ^2 test for dichotomized variables, Wilcoxon's rank test for paired variables and the Mann-Whitney test for independent variables applying Fisher's exact testing for significance.

Partitioning of gene expression levels to construct prognostic groups was performed according to LeBlanc *et al*^[41]. Briefly, the best cut-off value for a supposed

Table 1 Results of non-parametric paired samples Wilcoxon's test. mRNA expression of c-erbB-1 and c-erbB-2 are compared between tumor (T) and normal (N) samples

| Tumor | | c-erbB-1 | P | c-erbB-2 | P |
|--------------------------------|-----|----------|------|----------|-------|
| Pancreatic cancer | T<N | 21 | 0.03 | 14 | 0.99 |
| | T>N | 9 | | | |
| Cancer of the papilla of Vater | T<N | 5 | 0.38 | 6 | <0.05 |
| | T>N | 3 | | | |

prognostic variable is determined by simulating the log-rank test for all observed covariate values within the entire data set. The minimal log-rank P -value determines the best cut-off value for dichotomization of the covariate. Kaplan-Meier^[42] plots were used to describe the survival distribution and the log-rank test was used to evaluate survival differences^[43].

The level of significance was set at $P<0.05$ in all statistical testing. Unless otherwise specified, P -values were given for two-sided testing.

RESULTS

Survivin mRNA expression

Survivin mRNA expression was detected in 30 of 31 (97%) PC in all tumor specimens of the papilla of Vater and in all paired non-malignant control specimens. In 26/31 (83%) PC expression of survivin mRNA was higher in tumor compared to normal control tissue and this difference was statistically significant (Wilcoxon's test: $P<0.001$). In CPV, all tumor specimens ($n=8$) had a higher expression than the normal tissue (Wilcoxon's test: $P<0.01$). Expression of survivin mRNA was not significantly different between PC and CPV (Mann-Whitney test: $P=0.195$). Representative box-plots are shown in Figure 1.

C-erbB-1 mRNA expression

C-erbB-1 mRNA expression could be detected in 30 of 31 (97%) pancreatic tumors, all tumors of the papilla and all normal tissues. Expression of c-erbB-1 was lower in the malignant than in the normal pancreatic tissue in 21 out of 31 (68%) individual cases. This difference was statistically significant (Wilcoxon's test: $P=0.028$), whereas in CPV no statistically significant difference ($P=0.38$) in c-erbB-1 expression between tumor and corresponding normal tissue was revealed (Table 1).

C-erbB-2 mRNA expression

The expression of c-erbB-2 was detectable in all tumor and non-malignant specimens. In PC paired non-parametric testing revealed no statistically significant difference in c-erbB-2 expression between tumor and normal tissue. In contrast, c-erbB-2 expression was significantly (Wilcoxon's test: $P<0.05$) downregulated in 6/8 cases of CPV (Table 1).

No significant association between gene expression levels and clinical parameters such as sex (χ^2 test), tumor stage, lymph node stage, and grade of differentiation (Mann-Whitney test) was observed.

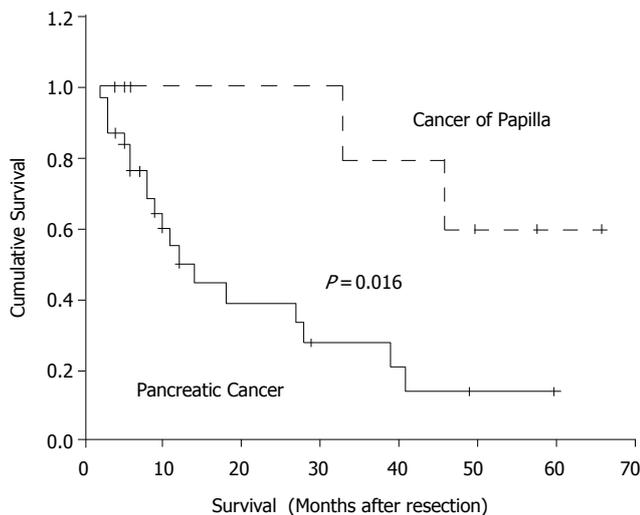


Figure 2 Kaplan-Meier analysis of survival of patients with pancreatic cancer ($n=31$) and CPV ($n=8$) after resection.

mRNA expression and survival of patients with pancreatic cancer and cancer of the ampulla of Vater

At a median follow-up of 56 months (range 7-89 mo) for surviving patients, median survival was 14 mo (range 2-60 mo) for patients with pancreatic carcinoma and 39.5 months (range 4-66 mo) for patients with carcinoma of the papilla of Vater (log-rank: $P<0.016$). Kaplan-Meier survival curves are shown in Figure 2.

Partitioning of gene expression levels to construct prognostic groups according to LeBlanc *et al*^[41] did not reveal any correlation between gene expression (c-erbB-1, c-erbB-2, and survivin) and survival of patients with pancreatic or papillary cancer.

DISCUSSION

The surgical therapy of carcinoma of the papilla of Vater and adenocarcinoma of the pancreas consists of a pylorus preserving or a classical pancreaticoduodenectomy (Whipple procedure). Despite this identical surgical approach, the long-term prognosis is different. Five-year survival rates from 0% to 25% for PC and from 15% to 56% for CPV have been reported^[1,12]. It was shown that different tumor biology, represented by higher expression of members of the EGF-R family (c-erbB-1, c-erbB-2, and c-erbB-3) contributes to the different behavior^[16,44]. C-erbB-1 and c-erbB-2 were also differentially expressed in our study. In contrast to Friess *et al*^[16], our results showed a significant downregulation of c-erbB-1 expression in PC tissue and of c-erbB-2 expression in the malignant tissue of the papilla of Vater compared with the adjacent normal tissues. These results were based on a smaller number of patients, but were obtained by quantitative real time RT-PCR. Whereas the above mentioned study^[16] applied immunohistochemical staining and Northern blot analysis, quantitative real time RT-PCR provides more accurate and reproducible quantitation of gene expression and has a large range of results^[45,46].

The downregulation of c-erbB-1 in malignant pancreatic tissues compared with the adjacent normal tissue is in contrast to the existing literature that shows

an overexpression of c-erbB-1 in pancreatic ductal adenocarcinoma and a worse prognosis for patients with high expression of c-erbB-1 in their tumors^[29,30,47,48]. As mentioned above, these studies, which were mainly published about 10 years ago, were based on immunohistochemical techniques showing c-erbB-1 expression in only 30-70% of the investigated PC cases. Importantly, the results were not compared with the matching normal pancreatic tissue from the same patients. In our investigation using real time RT-PCR, c-erbB-1 expression was detected in 97% of PCs and in all matching normal pancreatic samples.

The reported expression of c-erbB-2 in PC has varied widely (7-82%) in different studies due to the differences in methodology and/or patient selection^[35-38]. Our study, which is the first in using quantitative real-time RT-PCR to assess the expression of c-erbB-2 in pancreatic and CPV, did not show a different expression of c-erbB-2 between tumor and normal pancreatic tissue. This is in agreement with two recent studies^[49,50] using immunohistochemical methods, which found no correlation between c-erbB-2 expression and stage or survival of patients with PC. However, a series of other publications^[45-48,51] reported overexpression of c-erbB-2 in a subset of patients with PC and its prognostic relevance. For other malignancies such as gastric cancer^[52], breast cancer^[53], and ovarian cancer^[54] an increased expression of the c-erbB-2 oncogene has been reported, applying PCR based methods. According to our results and the recent literature one must consider, that the role of c-erbB-2 in the tumorigenesis and its value as a prognostic marker in PC has not been finally elucidated.

In concordance with other authors^[24,55-57], we found an increased expression of survivin in the samples of PC and CPV compared with the adjacent non-malignant pancreas. One study^[24] also found an overexpression of survivin in a series of 12 ampullary carcinomas; expression was not different from PC, which is comparable with our result. Kami *et al*^[58] reported a significant correlation between poor survival and increased expression of survivin in a study with 47 patients with PC using immunohistochemistry. Whereas Sarella *et al*^[24], as well as our study revealed no association between survival and expression of survivin. The comparable results of survivin expression in our patients and other studies in the literature strengthen our data regarding the expression of c-erbB-1 and c-erbB-2 in PC and CPV. For a variety of other malignancies it was shown that survivin expression is a reliable marker for an unfavorable disease with poor overall survival^[59,60]. The high number of locally advanced tumors, on which our and other studies are based, could explain the difference to PC. The significantly increased expression of survivin in PC, obtained by different methodologies, opens possibilities for new diagnostic and therapeutic strategies, such as new targets for gene therapy, differentiation between malignant and benign pancreatic lesions before surgical resection and to monitor response to neoadjuvant treatment regimens.

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Polymorphisms in interleukin-10 gene according to mutations of *NOD2/CARD15* gene and relation to phenotype in Spanish patients with Crohn's disease

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and the IL-10G14 microsatellite allele is associated with previous history of appendectomy and smoking habit at diagnosis. These data provide further molecular evidence for a genetic basis of the clinical heterogeneity of CD.

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Key words: Crohn's disease; *NOD2/CARD15* gene; Interleukin-10 gene

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Abstract

AIM: To examine the contribution of interleukin-10 (IL-10) gene polymorphisms to Crohn's disease (CD) phenotype, and the possible genetic epistasis between IL-10 gene polymorphisms and *CARD15/NOD2* gene mutations.

METHODS: A cohort of 205 Spanish unrelated patients with Crohn's disease recruited from a single center was studied. All patients were rigorously phenotyped and followed-up for at least 3 years (mean time, 12.5 years). The clinical phenotype was established prior to genotyping.

RESULTS: The correlation of genotype-Vienna classification groups showed that the ileocolonic location was significantly associated with the -1082G allele in the *NOD2/CARD15* mutation-positive patients ($RR = 1.52$, 95%CI, 1.21 to 1.91, $P = 0.008$). The multivariate analysis demonstrated that the IL-10 G14 microsatellite allele in the *NOD2/CARD15* mutation positive patients was associated with two risk factors, history of appendectomy ($RR = 2.15$, 95%CI = 1.1-4.30, $P = 0.001$) and smoking habit at diagnosis ($RR = 1.29$, 95%CI = 1.04-4.3, $P = 0.04$).

CONCLUSION: In Spanish population from Madrid, in CD patients carrying at least one *NOD2/CARD15* mutation, the -1082G allele is associated with ileocolonic disease

INTRODUCTION

Crohn's disease (CD) is a chronic inflammatory disorder of the gastrointestinal tract. The inflammation may involve any segment of the digestive tract, from the mouth to the anus, and may affect mucosa and deeper layers of the digestive wall, with or without granulomas. The etiopathogenesis of the disease remains poorly understood. Experimental and observational data suggest that intestinal inflammation arise from abnormal immune reactivity to bacterial flora in the intestine of individuals who are genetically susceptible^[1].

Epidemiologic and linkage studies suggest that genetic factors play a significant role in determining CD susceptibility. CD has no simple Mendelian pattern of inheritance. As other immune diseases, CD is thought to be a heterogeneous, complex polygenic disease, where both genetic and environmental factors play an important role in the disease and, in which multiple interactions between susceptibility and resistance alleles are involved in disease pathogenesis^[2,3].

Human genetic studies, notably the landmark identification in 2001 of *NOD2/CARD15* within the linkage region IBD1, have confirmed a genetic influence on CD^[4-6], and it is now clear that a genotype-phenotype relationship exists. In our population of Spanish CD

patients from Madrid, mutations in the *NOD2/CARD15* gene were a marker of susceptibility to disease and were associated with ileal disease^[7].

In CD, the mucosal inflammation is associated with an exaggerated and prolonged immune response because of a dysregulated production and interaction of pro-inflammatory and anti-inflammatory cytokines and their receptors^[8]. A variety of genes encoding various proteins involved in the immune regulation have been postulated as possible candidates for disease susceptibility, including, among others, cytokines as interleukin-10 (IL-10). IL-10 is a regulatory cytokine that has several functions, but one important role is to act as an inhibitor of development of Th1 cells, activated macrophages and their products interleukin-12 (IL-12), tumor necrosis factor (TNF) and interferon-gamma (IFN- γ). Even though it is usually considered an inhibitory cytokine, it also has stimulatory effects (e.g. stimulating B cell proliferation)^[9]. Recently, we have also shown that IL-10 polymorphisms contribute to susceptibility to CD in our Spanish population. IL-10G14 microsatellite allele as well as -1082G allele (guanine at position -1082) were significantly increased in Crohn's disease patients. The combined presence of both alleles in one individual notably increased the risk to develop CD^[10].

Although the pathogenetic mechanisms mediated by *NOD2/CARD15* remain elusive, it has recently been shown that one of the mutations in the *NOD2/CARD15* gene results in defective release of IL-10 from blood mononuclear cells after stimulation with Toll-like receptor (TLR) 2 ligands and this could contribute to the overwhelming inflammation seen in CD^[11].

As IL-10 polymorphisms appear to confer a risk to develop CD in the Spanish population, the present study examined genotype-phenotype correlations in the disease process. Moreover, after stratifying the patients on the basis of the presence or absence of the well-established *NOD2/CARD15* mutations, we looked for susceptibility factors being present in one specific phenotypic subpopulations.

MATERIALS AND METHODS

Study population

We studied a cohort of 205 Caucasian unrelated consecutive patients with CD who were recruited in a Unit of Inflammatory Bowel Disease (IBD) from a single tertiary referral center in Madrid, Spain. Diagnosis of Crohn's disease was based on standard clinical, radiologic, endoscopic, and histologic criteria^[12]. Phenotypic details were obtained by review of clinical charts and personal interview with the patients. The same clinical questionnaire was completed for each patient. This questionnaire included: date of birth, sex, familial IBD, age at diagnosis, follow-up interval, smoking habits, history of surgery (tonsillectomy, appendectomy), definitions of the Vienna classification for age at diagnosis (A1, < 40 years; A2, \geq 40 years), disease location (L1, terminal ileum; L2, colon; L3, ileocolon; L4, upper gastrointestinal), behavior (B1, nonstricturing nonpenetrating; B2, stricturing; B3, penetrating), perianal disease (defined as the presence of perianal abscess, fistulas and/or ulceration), extraintestinal

clinical manifestations (articular and cutaneous), and previous treatment as an indication of severity of disease (surgical intervention, corticosteroids, immunosuppressant agents, infliximab). All patient data were recorded by a gastroenterologist from the Unit of IBD (J. L. M.) who was blind to the genotype status of each patient. The protocol was approved by the Ethics Committee of the Hospital Clínico San Carlos, Madrid, and all patients were included in the study after giving informed consent.

Genotyping

IL-10 polymorphisms: IL-10G and IL-10R microsatellites were amplified using primers and conditions as previously described^[13]. Blood samples were subsequently denatured and run on an ABI Prism 3100 automatic sequencer (Applied Biosystems, Foster City, CA, USA). Each sample included an internal size standard (HD400 ROX, Applied Biosystems) in order to achieve a highly consistent measure. The results were analyzed using GeneMapper v3.0 (Applied Biosystems).

As previously described^[14], a combined amplification of the IL-10G microsatellite and the -1082 and -819 SNPs was performed. Our typing method allowed us to construct haplotypes directly. SNPs at positions -1082, -819 and -592 only form three different haplotypes in our population^[15,16], namely, ACC, ATA and GCC. Based on this previous finding, we only typed the samples for the two first SNPs, as the information provided by the third one is redundant.

NOD2/CARD15 polymorphisms: SNP13 (Leu1007fsinsC) was genotyped using a TaqMan assay (Applied Biosystems, Foster City, CA, USA). Primers and probes used were as previously described^[5,17] and PCR products were analysed on an ABI 7700 Sequence Detector (Applied Biosystems). SNP8 (Arg702Trp) (sense, 5'-CAT CTG AGA AGG CCC TGC TC(C/T)-3'; antisense, 5'-CAG ACA CCA GCG GGC ACA-3') and SNP12 (Gly908Arg) (sense, 5'-TTG GCC TTT TCA GAT TCT GG (G/C)-3'; antisense, 5'-CCC CTC GTC ACC CAC TCT G-3') were typed by allele-specific PCR. Detection of wild-type/mutant variants was assessed in an ABI 7700 Sequence Detector by an SYBRGreen assay. Previously sequenced samples were used as controls. In cases of doubt, samples were sequenced to confirm the result.

Statistical analysis

The frequencies for the IL-10 polymorphisms and *NOD2/CARD15* mutations were estimated by counting gene and calculating sample proportions. Subsequently, Hardy Weinberg equilibrium for each of the polymorphisms was tested to check for Mendelian inheritance using χ^2 test with one degree of freedom. Carrier status was considered if any subject inherited at least one copy of the mutant allele. The association between IL-10 polymorphisms and phenotypic characteristics of CD was estimated by the relative risk (RR) with the 95% confidence interval (CI). Logistic regression analysis was performed to assess whether IL-10 polymorphisms were correlated with a particular clinical phenotype. The multiple logistic regression analysis was

Table 1 Distribution of IL-10G14 microsatellite allele and -1082G allele stratified by NOD2/CARD15 status

| Allele frequencies | At least one CARD15/NOD2 mutation positive <i>n</i> = 76 (%) | CARD15/NOD2 mutation negative <i>n</i> = 129 (%) | <i>P</i> |
|----------------------------------|---|---|----------|
| IL-10G14 (<i>n</i> = 47) | 22 (28.9) | 25 (19.4) | 0.115 |
| -1082G (<i>n</i> = 146) | 54 (71.1) | 92 (71.3) | 0.96 |
| IL-10G14+-1082G (<i>n</i> = 29) | 13 (17.1) | 16 (12.4) | 0.35 |

Table 2 Distribution of -1082G allele among different clinical subgroups of CD stratified by NOD2/CARD15 status

| Phenotypic characteristics | Phenotype frequency of -1082 IL-10G(+) (<i>n</i> = 146) (%) | | CARD15/NOD2 (+) -1082 IL-10 G(+) (<i>n</i> = 54) (%) | | CARD15/NOD2 (-) -1082 IL-10 G(+) (<i>n</i> = 92) (%) | |
|---|---|----------|--|----------|--|----------|
| | | <i>P</i> | | <i>P</i> | | <i>P</i> |
| Sex | | | | | | |
| Men | 67 (45.9) | | 24 (44.4) | 0.77 | 43 (46.7) | 0.45 |
| Women | 79 (54.1) | 0.67 | 30 (55.5) | | 49 (53.3) | |
| Age at diagnosis (yr) | | | | | | |
| A1, < 40 | 117 (80.1) | | 46 (85.2) | 0.5 | 71 (77.2) | 0.41 |
| A2, ≥40 | 29 (19.9) | 0.72 | 8 (14.8) | | 21 (22.8) | |
| Family history | 27 (18.5) | 0.43 | 10 (18.5) | 0.43 | 17 (18.5) | 0.76 |
| Smokers | 64 (43.8) | 0.55 | 24 (44.4) | 0.86 | 40 (43.5) | 0.65 |
| Appendectomy | 20 (13.7) | 0.55 | 7 (12.9) | 0.93 | 13 (14.1) | 0.49 |
| Tonsillectomy | 22 (15.1) | 0.11 | 5 (9.3) | 0.66 | 17 (18.5) | 0.19 |
| Disease behavior | | | | | | |
| Nonstricturing, nonpenetrating (B1) | 63 (43.2) | 0.87 | 24 (44.4) | 0.58 | 39 (42.4) | 0.86 |
| Stricturing (B2) | 22 (15.1) | | 9 (16.7) | | 13 (14.1) | |
| Penetrating (B3) | 61 (41.8) | | 21 (38.9) | | 40 (43.5) | |
| Location of disease | | | | | | |
| Terminal ileum (L1) | 70 (47.9) | 0.63 | 29 (53.7) | 0.021 | 41 (44.6) | 0.43 |
| Colon (L2) | 24 (16.4) | | 5 (9.3) | | 19 (20.7) | |
| Ileocolon (L3) | 47 (32.2) | | 19 (35.2) | | 28 (30.4) | |
| Upper gastrointestinal (L4) | 5 (3.4) | | 1 (1.9) | | 4 (4.3) | |
| Perianal | 39 (26.7) | 0.30 | 11 (20.4) | 1 | 28 (30.4) | 0.3 |
| Extraintestinal clinical manifestations | | | | | | |
| Cutaneous | 27 (18.5) | 0.56 | 9 (16.7) | 0.29 | 18 (19.6) | 0.93 |
| Articular | 49 (33.6) | 0.69 | 16 (29.6) | 0.83 | 33 (35.9) | 0.83 |
| Treatment | | | | | | |
| Surgical intervention | 61 (41.8) | 0.76 | 22 (40.7) | 0.71 | 39 (42.4) | 0.92 |
| Infliximab | 19 (13.0) | 0.06 | 5 (9.3) | 0.032 | 14 (15.2) | 0.61 |
| Immunosuppressants | 63 (43.2) | 0.81 | 25 (46.3) | 0.72 | 38 (41.3) | 0.63 |

¹ -1082G allele in NOD2/CARD15 mutation positive patients: ileocolonic location ($P=0.008$, $RR=1.52$, $95\%CI=1.21-1.91$). ² -1082G allele in NOD2/CARD15 mutation positive patients: infliximab ($P=0.03$, $RR=0.54$, $95\%CI=0.27-1$)

adjusted for age (years). A two-tailed P value equal to or less than 0.05 was considered statistically significant. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 10.07 for Windows (SPSS Inc., Chicago, Ill. USA).

RESULTS

The cohort of 205 patients with Crohn's disease consisted of 109 men and 96 women. The median age at diagnosis was 27 years (mean 31.6, range 8-80) with an interquartile range of 22-26 years. The median duration of follow-up was 11 years (mean 12.57, range 3-47) with an interquartile range of 7-16 years.

No statistically significant difference in IL-10G14 and -1082G or both allele frequencies was found between NOD2/CARD15 mutation positive and negative patients (Table 1).

The genotype-phenotype correlations are shown in Tables 2 and 3. No Vienna classifications of the disease, neither risk factors, clinical manifestations, or treatment modalities were associated with either IL-10G14 or -1082G alleles. A gene-dosage effect (IL-10G14 and -1082G alleles) on phenotypic characteristics was not observed (data

not shown).

When we examined the associated IL-10G14 and -1082G alleles in the NOD2/CARD15 mutation positive and negative patients separately, three new positive associations were found. With regard to Vienna classifications of the disease, ileocolonic disease was significantly associated with -1082G allele in the NOD2/CARD15 mutation positive patients ($P=0.008$, Table 2). On the other hand, relative to risk factors for Crohn disease, two significant associations of the IL-10G14 allele carriership and history of appendectomy ($P=0.002$) and smoking habit at diagnosis ($P=0.02$) in the NOD2/CARD15 mutation positive patients as compared with the negative patients were observed (Table 3). The multivariate analysis demonstrated that IL-10G14 allele was associated with history of appendectomy ($P=0.001$, $RR=2.15$, $95\%CI=1.1-4.30$) and with smoking habit at diagnosis ($P=0.04$, $RR=1.29$, $95\%CI=1.04-4.3$).

DISCUSSION

In this study, we performed a genotype-phenotype correlation study in a cohort of 205 Caucasian patients with Crohn's disease from the community of Madrid

Table 3 Distribution of IL-10G14 allele among the different clinical subgroups of CD stratified by NOD2/CARD15 status

| Phenotypic characteristics | Phenotype frequency of IL-10.G14 (+) (n = 47) (%) | | CARD15/NOD2 (+) IL-10.G14, (n = 22) (%), | | CARD15/NOD2 (-) IL-10.G14, (n = 25) (%), | |
|---|---|------|--|--------------------|--|------|
| | | P | | P | | P |
| Sex | | | | | | |
| Men | 23 (48.9) | 0.74 | 13 (59.1) | 0.08 | 10 (40) | 0.32 |
| Women | 24 (51.1) | | 9 (40.9) | | 15 (60) | |
| Age at diagnosis (yr) | | | | | | |
| A1, < 40 | 39 (83.0) | 0.5 | 21 (95.5) | 0.27 | 18 (72) | 0.68 |
| A2, ≥40 | 8 (17.0) | | 1 (4.5) | | 7 (28) | |
| Family history | 9 (19.1) | 0.54 | 4 (18.2) | 0.52 | 5 (20) | 0.88 |
| Smokers | 24 (51.1) | 0.51 | 16 (72.7) | 0.02 ¹ | 8 (32) | 0.41 |
| Appendectomy | 9 (19.1) | 0.32 | 6 (27.3) | 0.002 ² | 3 (12) | 0.59 |
| Tonsillectomy | 5 (10.6) | 0.63 | 3 (13.6) | 0.48 | 2 (8) | 0.36 |
| Disease behavior | | | | | | |
| Nonstricturing, nonpenetrating (B1) | 19 (40.4) | 0.61 | 6 (27.3) | 0.19 | 13 (52) | 0.62 |
| Stricturing (B2) | 9 (19.1) | | 6 (27.3) | | 3 (12) | |
| Penetrating (B3) | 19 (40.4) | | 10 (45.5) | | 9 (36) | |
| Location of disease | | | | | | |
| Terminal ileum (L1) | 23 (48.9) | 0.26 | 15 (68.2) | 0.54 | 8 (32) | 0.24 |
| Colon (L2) | 4 (8.5) | | 1 (4.5) | | 3 (12) | |
| Ileocolon (L3) | 17 (36.2) | | 5 (22.7) | | 12 (48) | |
| Upper gastrointestinal (L4) | 3 (6.4) | | 1 (4.5) | | 2 (8) | |
| Perianal | 14 (29.8) | 0.86 | 4 (18.2) | 0.47 | 10 (40) | 0.28 |
| Extraintestinal clinical manifestations | | | | | | |
| Cutaneous | 8 (17.0) | 0.62 | 3 (13.6) | 0.30 | 5 (20) | 0.56 |
| Articular | 13 (27.7) | 0.32 | 6 (27.3) | 0.53 | 7 (28) | 0.36 |
| Treatment | | | | | | |
| Surgical intervention | 20 (42.6) | 0.98 | 10 (45.5) | 0.8 | 10 (40) | 0.82 |
| Infliximab | 7 (14.9) | 0.79 | 5 (22.7) | 0.31 | 2 (8) | 0.21 |
| Immunosuppressants | 18 (38.3) | 0.61 | 10 (45.5) | 0.81 | 8 (32) | 0.23 |

¹IL-10G14 in NOD2/CARD2 mutation positive patients: smokers vs non-smokers ($P=0.02$, $RR=2.47$, $95\%CI=1.28-4.8$). ²IL-10 G14 in NOD2/CARD2 mutation positive patients: appendectomy vs non-appendectomy ($P=0.002$, $RR=3.29$, $95\%CI=1.45-7$)

(central Spain) who had been followed-up for a mean of 12.57 years. The clinical diagnosis of Crohn's disease was confirmed by the criteria of Gasche *et al*^[18]. Our results showed that a relation existed between disease location (ileocolon), risk factors for CD (appendectomy and smoking habit) and genetic heterogeneity in our population. This could suggest an epistatic interaction of both genes.

CD is an extensively heterogeneous disease. Epidemiologic and genetic data suggest that heterogeneity of CD may be genetically determined. Recently, Ahmad *et al*^[19] have shown the importance of the *NOD2/CARD15* gene and the HLA region in determining clinical subgroups of CD. Similarly, in our CD population, we confirmed the association between *NOD2/CARD15* mutations and ileal disease and the strong association between DRB1*0103 allele and colonic disease^[17]. These studies may provide an initial basis for the construction of a molecular classification of CD^[20].

Location of disease is the variable that remains more stable during the course of the disease^[21] and it showed a stronger association with mutations of the *NOD2/CARD15* gene in genotype-phenotype studies using the Vienna classification. In our population^[7], like others^[19,22,23], possession of an *NOD2/CARD15* variant was significantly associated with ileal disease. On the contrary, mutations of the *NOD2/CARD15* gene were exceptional in patients with solely colonic involvement^[7]. In contrast to findings in the Norwegian and German populations^[24] and similar to findings in the British population^[19,25], we could not find an

association between ileocolonic location and mutations of the *NOD2/CARD15* gene^[7]. In contrast, this association has been found between the *NOD2/CARD15* variants and IL-10 -1082G carriers. This suggests the importance of classifying the patients according to the different genes implicated in the etiopathogenesis of CD and, therefore, of performing the molecular characterization of CD patients. Tagore *et al*^[26] have shown that IL-10 production is associated with three biallelic polymorphisms within the promoter region of the IL-10. The allele -1082G is associated with higher IL-10 production in peripheral blood leukocytes. This different levels of IL-10 expression could explain the diverse phenotypic clinical behaviour of CD in specific genetic susceptibility background, like *NOD2/CARD15*.

No other factor is likely to contribute in isolation to the pathogenesis of CD. The modulation of the immune system either locally or systemically has an important role to play in the etiology and pathogenesis of this disease. The IL-10 gene has special interest for a candidate gene approach in CD. The biology of IL-10 is highly complex: a potent down-regulator of CD peripheral monocyte and intestinal lamina propria mononuclear cells activator *in vitro* and *in vivo*^[27]. Both human IL-10 and murine IL-10 exert immunostimulatory effects by up-regulating MHC class II expression in B lymphocytes and inducing cytotoxic T-cell differentiation^[28]. Due to the dual regulatory function of IL-10 itself, its gene is indeed an interesting susceptibility candidate and it would be worthwhile to know whether environment factors could modulate the IL-10 function.

The IL-10 gene knockout mouse spontaneously develops a chronic enterocolitis^[29] and gene therapy using an adenovirus IL-10 construct is successful in preventing experimental colitis in rats^[30]. Moreover, there have been preliminary reports of amelioration of clinical symptoms of CD following administration of human recombinant IL-10^[27,31].

Regarding the risk factors for CD, we found two significant associations between carriage of the IL-10G14 microsatellite allele in CARD15-positive patients and previous history of appendectomy and smoking habit at diagnosis. The interaction between genetics and smoking has been demonstrated in siblings from mixed-disease families, where some individuals develop CD and others in the same family develop ulcerative colitis. There is a strong positive relationship between smoking and CD and an equally strong negative relationship between smoking and ulcerative colitis^[32]. Appendectomy provides a spectrum of protection against ulcerative colitis development and progression, whereas its role in CD remains unclear. Russel *et al.*^[33] have also noted a positive association of CD and previous appendectomy, suggesting that, in some cases, appendectomy is a result of still undiagnosed CD. Recently, other retrospective study concluded the risk of CD after appendectomy was associated with an increased risk of CD dependent on the patient's sex, age, and the diagnosis at operation^[34]. Future work should pursue to investigate the epidemiological relationships in CD, addressing a greater number of potentially important confounders, such as smoking, hygiene, and pathology of the appendix. And parallel with these clinical observations, new target could be defined by genetic and immunologic analysis to evaluate if appendicitis could be correlated with any particular genetic modification involved for patients with CD^[35].

In conclusion, our study has shown that in the Spanish population from Madrid, in CD patients carrying at least an *NOD2/CARD15* mutant, the -1082G allele might be associated with ileocolonic disease, and the IL-10G14 microsatellite allele might be associated with previous history of appendectomy and smoking habit at diagnosis. Identification of plausible factors that may interact with genes is a promising step toward understanding how sequence variation influences disease susceptibility.

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Atrial fibrillation after surgery for esophageal carcinoma: Clinical and prognostic significance

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Abstract

AIM: To retrospectively evaluate the clinical relevance, perioperative risk factors, outcome of different pharmacological prophylaxis, and short-term prognostic value of atrial fibrillation (AF) after surgery for esophageal carcinoma.

METHODS: We retrospectively studied 63 patients with AF after surgery for esophageal carcinoma in comparison with 126 patients without AF after esophagectomy during the same time. Postoperative AF incidence was related to different clinical factors possibly involved in its occurrence and short-term survival.

RESULTS: A strong relationship was observed between AF and postoperative hypoxia, history of chronic obstructive pulmonary disease (COPD), postoperative thoracic-gastric dilatation, age older than 65 years, male sex and history of cardiac disease. No difference was observed between the two groups with regard to short-term mortality and length of hospital stay.

CONCLUSIONS: AF occurs more frequently after esophagectomy in aged and male patients. Other factors contributing to postoperative AF are history of COPD and cardiac disease, postoperative hypoxia and thoracic-gastric dilatation.

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Key words: Esophageal carcinoma; Atrial fibrillation; Surgery

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INTRODUCTION

Atrial fibrillation (AF) is a frequently occurring arrhythmia after esophageal procedures. It has been suggested that AF may be generally related to a worse prognosis and a longer postoperative hospital stay^[1]. Reports on the effectiveness of alternative strategies to control AF are contradictory^[2,3]. Therefore identification of all high-risk populations will allow targeted use and hence more cost-effective and successful application of these methods can be achieved.

The purpose of the present study was to retrospectively evaluate the clinical relevance, perioperative risk factors, outcome of different pharmacological interventions, and short-term prognostic value of AF after surgery for esophageal carcinoma.

MATERIALS AND METHODS

Patients

From 1998 to the end of 2003, a total of 63 consecutive patients had AF after surgery for esophageal carcinoma in our institution. AF was defined as a sustained or repetitive electrocardiographically documented arrhythmia requiring antiarrhythmic therapy^[4]. During the same time of surgery, 126 patients without postoperative AF after esophageal carcinoma resection were randomly chosen as the control group. A retrospective chart review was performed on all the patients with and without AF. Patients were identified by search of registry database and hospital medical records. Records were reviewed by two independent researchers to confirm the diagnosis of AF.

There were 63 AF patients, 55 males and 8 females with an average age of 64.7 (range, 51-83) years. The onset of AF occurred on the first postoperative day in 22 patients (34.9%), on the second postoperative day in 30 patients (47.6%), on the third postoperative day in 7 patients (11.1%), and on the fourth postoperative day, and later in 4 patients (6.4%). The control group consisted of 126 patients, 94 males, and 32 females with an average age of 57.2 (range, 32-79) years. Patients with a known history of atrial dysrhythmias or those receiving digoxin were excluded from analysis.

Methods

Postoperative AF incidence was related to different pre-

Table 1 Preoperative factors associated with postoperative AF (%)

| Group | Age older than 65 years | Male sex | Cardiac diseases | COPD | Hypertension | Diabetes mellitus |
|---------|-----------------------------|----------------------------|----------------------------|----------------------------|--------------|-------------------|
| AF | 39.68 (25/63) ^b | 87.30 (55/63) ^a | 19.05 (12/63) ^a | 46.03 (29/63) ^b | 9.52 (6/63) | 14.29 (9/63) |
| Control | 21.43 (27/126) ^b | 74.60 (94/126) | 7.14 (9/126) | 11.11 (14/126) | 5.56 (7/126) | 12.70 (16/126) |

^a $P < 0.05$, ^b $P < 0.01$ vs control group.

Table 2 Intraoperative and postoperative factors associated with postoperative AF (%)

| Group | Right thorax approach | Anastomosis at the neck | Anastomosis below the aortic arch | Anastomosis above the aortic arch | Hypotension in operation | Postoperative fever | Postoperative hypoxia | Thoracic-gastric dilatation |
|---------|-----------------------|-------------------------|-----------------------------------|-----------------------------------|--------------------------|---------------------|----------------------------|-----------------------------|
| AF | 11.11 (7/63) | 22.22 (14/63) | 14.29 (9/63) | 63.49 (40/63) | 7.94 (5/63) | 14.29 (9/63) | 25.40 (16/63) ^b | 31.75 (20/63) ^b |
| Control | 7.94 (10/126) | 11.90 (15/126) | 10.32 (13/126) | 77.78 (98/126) | 7.14 (9/126) | 8.73 (11/126) | 4.76 (6/126) ^b | 18.25 (23/126) ^b |

^b $P < 0.01$ vs control group.

operative, intraoperative, and postoperative clinical factors possibly involved in its occurrence and short-term survival. The demographic data that were analyzed included patients' age, sex, history of cardiac disease, chronic obstructive pulmonary disease (COPD, defined as $FEV_1 \leq 70\%$ predicted and FEV_1/FVC ratio $\leq 70\%$ in pulmonary function tests), diabetes mellitus, and hypertension. Intraoperative and postoperative factors examined were surgical approach (via the left or the right thorax), site of anastomosis (at the neck, above or below the aortic arch), hypotension in operation (defined as systolic blood pressure lower than 6 kPa, persisting for more than 5 min), postoperative fever (defined as body temperature higher than 38.5°C , persisting for more than 3 d), hypoxia (defined as aortic blood oxygen saturation lower than 93%, persisting for more than 1 h before the onset of AF), thoracic-gastric dilatation before the onset of AF confirmed by chest X-ray, 30-d mortality rate and length of hospital stay.

Statistical analysis

Statistical analysis was performed with the SPSS software version 10.0. Intergroup difference was determined using Student's *t* test and Fisher's exact test. χ^2 test was used to analyze categorical data. To identify which factors could predict AF, univariate, and stepwise multiple logistic regression analysis was used. $P < 0.05$ was considered statistically significant.

RESULTS

A higher incidence of AF was found in male patients older than 65 years, with a history of cardiac diseases and COPD than that in the control group patients (Table 1). There was no significant difference in patients with a history of hypertension and diabetes mellitus between the two groups. AF patients had a higher incidence of postoperative hypoxia and thoracic-gastric dilatation than the control group (Table 2). There was no significant difference in surgical approach, anastomosis site, intraoperative hypotension and postoperative fever between the two groups.

Results from the univariate analysis for the association of each factor with AF are summarized in Table 3. The factors associated with increased risk for AF on multivariate analysis included postoperative hypoxia, history of COPD, thoracic-gastric dilatation, age older than 65 years, male sex and history of cardiac disease. The incidence of AF was not dependent on the history of hypertension and diabetes mellitus, intraoperative hypotension, surgical approach, anastomosis site, and postoperative fever.

In the AF group, 15 patients who did not receive any antiarrhythmic drug therapy in the early stage of AF were given oxygen and sedation therapy. Only one of the 15 patients (6.67%) had sinus rhythm spontaneously within 24 h. All the other 62 patients were treated with antiarrhythmic drugs such as cedilanid, isoptin, propafenone and amiodarone. It was considered successful if the heart rhythm changed to sinus rhythm in 24 h after the treatment. Otherwise, other antiarrhythmic drugs were used. The outcome of antiarrhythmic therapy is presented in Table 4.

Death within 30 d after the surgery occurred in 2 AF patients with a mortality of 3.2% (2/63), and in 2 patients of the control group with a mortality of 1.6% (2/126) ($P > 0.05$). The overall mortality in our patients was 2.1% (4/189). None of these deaths were directly attributed to AF. The average length of hospital stay was 10.65 ± 0.87 d for the AF group and 9.98 ± 0.96 d for the control group ($P > 0.05$).

DISCUSSION

AF after esophagectomy remains one of the most frequent complications. The cause for postoperative AF is unclear. However, previous studies have shown different results, sometimes contradictory with regard to the clinical significance of various factors for AF after esophagectomy^[1,3]. We found that postoperative hypoxia, history of COPD, thoracic-gastric dilatation, age older than 65 years, male sex, and history of cardiac disease were predictors of postoperative AF. We considered that only clinically significant

Table 3 Univariate analysis of risk factors for postoperative AF

| Risk factors | P |
|------------------------------|--------|
| Postoperative hypoxia | <0.001 |
| COPD | 0.001 |
| Thoracic-gastric dilatation | 0.009 |
| Age more than 65 y | 0.009 |
| Male sex | 0.017 |
| History of heart disease | 0.038 |
| Postoperative fever | 0.051 |
| History of hypertension | 0.062 |
| Surgical approach | 0.143 |
| Anastomosis site | 0.189 |
| History of diabetes mellitus | 0.412 |
| Intraoperative hypotension | 0.475 |

AF defined by electrocardiography had an association with hemodynamic changes. A possible limitation of our study is that continuous electrocardiographic monitoring was not employed to detect asymptomatic AF, as our study was designed to evaluate clinically significant AF, which was the focus of patients' complaints and needed therapeutic interventions. Our conclusions, therefore, do not apply to the occurrence of asymptomatic AF after the surgery for esophageal carcinoma.

We did not find differences in survival rate and length of hospital stay between patients who developed AF and those who did not in the early postoperative period. We found that some pharmacological interventions were effective for AF after esophagectomy, but symptomatic management without drug treatment was not effective, suggesting that AF, if treated promptly, is fairly tolerated in all the patients. The peak incidence of AF on the second day after surgery, with more than 80% of events occurring during the first three postoperative days, is consistent with the findings of a previous study^[6]. We speculate that AF after esophagectomy is precipitated by the resolution of inflammatory response following blunt or sharp surgical trauma to sympathovagal nerve fibers supplying the heart for 1-4 d postoperatively, which alter the autonomic modulation of atrial myocardial cells to endogenous catecholamines.

The identification of reliable predictors of AF after esophagectomy may help us to develop corresponding preventive strategies. The presence of postoperative hypoxia and history of COPD were associated with the development of AF in our study. It has been well established that due to diminished cardiopulmonary reserve, patients with COPD and hypoxia are more prone to perioperative complications following noncardiac thoracic surgical procedures in general^[7,8]. The definition of COPD might vary between different authors and our definition was based on the pulmonary function testing, and some authors' definition is based on the presence or absence of bronchodilator treatment^[4].

The association between postoperative thoracic-gastric dilatation and AF found in our analysis has not been previously demonstrated. We speculate that it may correlate with hypoxia, physical influence on the heart from the

Table 4 Outcome of AF after drug treatment

| Prophylaxis | Cases | Successful antiarrhythmic cases (%) |
|-------------|-------|-------------------------------------|
| Cedilanid | 51 | 18 (35.29) |
| Isoptin | 17 | 2 (11.76) |
| Propafenone | 21 | 11 (52.38) |
| Amiodarone | 20 | 20 (100.00) |

dilated stomach, and the uncomfortable feeling of patients for the thoracic-gastric dilation. It is not surprising that advanced age is a strong predictor of postoperative AF, because at the age of 75, only approximately 10% of normal sinus node pacemaker cells remain^[9]. It is well known that age-related cardiac structural changes, such as increased fibrous and adipose tissue in the sinoatrial node, and focal interstitial deposits of amyloid in the atria, and atrial dilations may play a significant role in the genesis of arrhythmia^[10]. The age is closely related with AF^[11].

The association between male sex and AF might be explained by the effect of sex on immune response. One hypothesis for the cause of postoperative AF is atrial or pulmonary vein inflammation^[12]. After trauma, male patients have an increased proinflammatory immune response compared to female patients^[13], and this might lead to increased AF after surgical trauma. The association of cardiac disease with AF has been demonstrated inconsistently by other investigators^[14]. The common occurrence of AF after cardiac surgery supports the association of cardiac disease with AF after thoracic surgery. The lack of association between hypertension and AF in our analysis is also noteworthy. Other investigators have shown this association, but their analysis is limited to patients with lung cancer^[15].

In conclusion, AF is associated with postoperative hypoxia, history of COPD, thoracic-gastric dilatation, age older than 65 years, male sex, and history of cardiac disease. The identification of patients with a high risk for AF will allow more direct application of pharmacological and alternative methods of prophylaxis.

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Expression of dendritic cell-specific intercellular adhesion molecule 3 grabbing nonintegrin on dendritic cells generated from human peripheral blood monocytes

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Abstract

AIM: To generate dendritic cells (DCs) from human peripheral blood and to detect the expression of dendritic cell-specific intercellular adhesion molecule 3 grabbing nonintegrin (DC-SIGN; CD209) for the further study of DC-SIGN in hepatitis C virus (HCV) transmission.

METHODS: Peripheral blood monocytes were isolated from blood of healthy individuals by Ficoll–Hypaque sedimentation and cultured in complete medium containing rhGM-CSF and rhIL-4. Cells were cultured for seven days, with cytokine addition every two days to obtain immature DCs. Characteristics of the cultured cells were observed under light and scanning microscope, and the expression of DC-SIGN was detected by immunofluorescence staining.

RESULTS: After seven-day culture, a large number of cells with typical characteristics of DCs appeared. Their characteristics were observed under light and scanning electron microscope. These cells had a variety of cell shapes such as those of bipolar elongate cells, elaborate stellate cells and DCs. DC-SIGN was detected by immunofluorescence staining and its expression level on cultivated dendritic cells was high.

CONCLUSION: DCs with a high expression of DC-SIGN can be generated from human peripheral blood monocytes in complete medium containing rhGM-CSF and rhIL-4.

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Key words: Expression of DC-SIGN; Dendritic cells; Peripheral blood monocytes.

eral blood monocytes.

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INTRODUCTION

Dendritic cells (DCs) are professional antigen presenting cells (APCs) critically involved in the initiation of T cell-dependent immune responses as a consequence of their high expression of MHC and co-stimulatory molecules. However, it is becoming increasingly clear that some pathogens subvert DC functions to escape immune surveillance^[1]. Many cell-surface molecules of DCs can bind to viral envelope glycoproteins without mediating entry. Instead, they serve as capture receptors that disseminate viral particles to target organs or susceptible cells. The C type lectin of DC-SIGN, one of the cell-surface molecules of DCs, functions as a capture receptor for several viruses, such as HIV type 1 (HIV-1)^[2], hepatitis C virus (HCV)^[3] and dengue virus^[4]. Therefore, further investigations of the function of DCs and the interactions between DC-SIGN and pathogens are helpful in determining their importance *in vivo*. In this study, we generated dendritic cells from peripheral blood mononuclear cells (PBMC) from normal donors and high DC-SIGN expression was detected on the surface of dendritic cells by immunofluorescence staining.

MATERIALS AND METHODS

Medium and reagents

RPMI1640 and fetal calf serum (FCS) were purchased from Gibco Laboratories (Grand Island, NY). FCS was heat-inactivated at 56°C for 30 min. Human recombinant IL-4(rhIL-4) and rhGM-CSF were from R&D Systems (Abingdon, UK) and used at 1000U/mL, respectively. Monoclonal anti-human DC-SIGN phycoerythrin (FAB161P) was also from R&D Systems. Bovine serum albumin (BSA) was from Huamei Co. Human peripheral

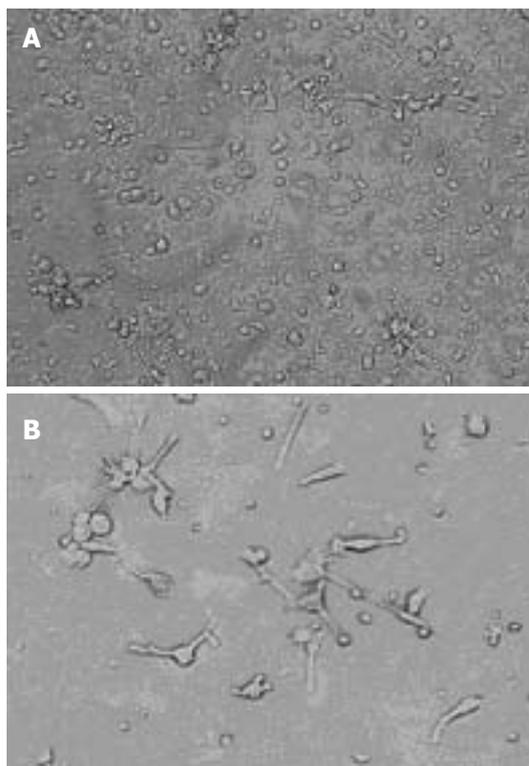


Figure 1 Dendritic cells in liquid cultures of complete RPMI 1640 supplemented with FCS, rhGM-CSF and rhIL-4. **A:** Low-power view of adherent PBMC after 3 d of culture (original magnification $\times 200$). Red arrows indicate the cellular aggregates and black arrows indicate veil- or sheet-like processes of dendritic cells; **B:** Veil- or sheet-like processes of most cells after 7 d of culture (original magnification $\times 400$).

blood was obtained from healthy adult donors.

Culture of DCs

Human peripheral blood mononuclear cells (PBMC) were isolated from buffer coat from normal donors by Ficoll-Hypaque density gradient centrifugation according to standard procedures. Monocytes were re-suspended in RPMI-10% FCS at 1×10^6 cells/mL, and allowed to adhere to 6-well plates (Costar Corp., Cambridge, MA). After a 2h adherence step at 37 °C in complete medium, nonadherent cells were washed with PBS and the remaining adherent cells were immediately subjected to the DC differentiation protocol. Briefly, monocytes were re-suspended and cultured in RPMI 1640 supplemented with 10% FCS, 25 mmol/L HEPES, and 2 mmol/L glutamine (complete medium) containing 1000 U/mL rhGM-CSF and 1000 U/mL rhIL-4. Cells were cultured for seven days, with cytokine addition every two days to obtain DCs (before incubation, a piece of 12mm \times 12mm sterile coverslip was put into one of the plates and used for scanning electronic microscopy)

Morphological observation of cultured cells

Cells were observed under light microscope during incubation. After seven-day culture, the coverslip was covered with DCs and prefixed with a 2% glutaraldehyde solution for 1 h at 4 °C. Post-fixation was done with a 2% osmium tetroxide solution for 30 min at 4 °C. After each fixation, the samples were washed twice in PBS containing



Figure 2 Cells cultivated with 10% FCS-RPMI 1640 supplemented with rhGM-CSF and rhIL-4 for 7 d (original magnification $\times 1500$).

BSA, dehydrated in graded ethanol and dried at “critical point” in liquid CO₂ under 95-bar pressure. Then, the coverslip was covered with gold by cathodic spraying. Finally, the samples were examined under scanning electron microscope (S-520, HITACHI).

Immunofluorescence staining of DC-SIGN

Cells to be used for staining with antibody were Fc-blocked cells by treatment with 1 μ g of BSA/ 10^5 cells for 15 min at room temperature, without washing the excess blocking BSA from this reaction. Then 25 μ L of the Fc-blocked cells (1×10^5 cells) was transferred to a 5 mL tube, and 10 μ L of PE-conjugated anti-DC-SIGN reagent was added. After incubated for 30-45 min at 4 °C in the dark, un-reacted anti-DC-SIGN reagent was removed by washing the cells twice in 4 mL of isotonic phosphate buffer supplemented with 0.5% BSA by centrifugation at 500 r/min for 5 min. The cell pellet was re-suspended in 100 μ L of PBS buffer and allowed to adhere to poly-L-lysine-coated coverslips for 30 min at 4 °C in darkness for final immunofluorescence. The representative fields of cells were photographed through an oil immersion lens on a Leica DM 6000B microscope (Leica Microsystems, Wetzlar, Germany).

RESULTS

Morphology of cultivated DCs

Phase-contrast observation: Three cellular aggregates were attached to a layer of adherent cells (Figure 1A). Some of the profiles in the aggregates had veil- or sheet-like processes of DCs. On d 7 of culture, these adherent

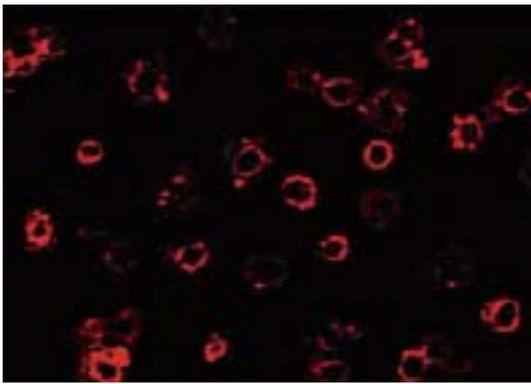


Figure 3 Cells stained with monoclonal anti-human DC-SIGN phycoerythrin (original magnification×400).

cells emigrated from the clusters, came off the surface and many typical DCs were seen floating in the culture medium (Figure 1B).

Scanning electron microscopy: Further morphological observations were carried out under scanning electron microscope (SEM). These cells had a variety of cell shapes such as bipolar elongated cells, elaborate stellate cells and DCs. Most pseudopods were long and uniform in width with blunt terminations, but smaller spinous processes were also evident (Figure 2). This phenomenon indicated that DCs had a variety of branching forms, and constantly extended and retracted many fine cell processes. The adjective “dendritic” might be appropriate for this particular cell type (Figure 3).

Determination of DC-SIGN expression

The DC-SIGN expression was detected by immunofluorescence staining with monoclonal anti-human DC-SIGN phycoerythrin. Most of the cultured cell surfaces were stained red, exhibiting a high level of expression of DC-SIGN on the surface of DCs.

DISCUSSION

In this study, we generated DCs from human PBMC with 10% FCS-RPMI 1640 supplemented with rhGM-CSF and rhIL-4. DC-SIGN expression was found on the surface of DCs.

DCs are specialized and co-stimulatory cells which play an important role in the induction of cellular immune response to antigens. DCs interact with pathogens using conserved pattern-recognition receptors, which recognize characteristic molecular arrangements within microbial carbohydrates, lipids and nucleic acids^[5]. Receptors of this type include Toll-like receptors (TLRs)^[6,7] and C-type lectins^[8]. TLRs can transfer information about the interacting pathogens into DCs through intracellular signaling cascades, thereby eliciting appropriate cellular processes, such as DC maturation and induction of anti-inflammatory cytokines^[5,6,9]. By contrast, C-type lectins recognize pathogens through their carbohydrate structure and internalize the pathogens for antigen processing and presentation^[10,11]. Pathogens survive and evade the antimicrobial defense system of the host by subverting the

function of these pattern-recognition receptors. Hence, an understanding of the interaction of pathogens with these receptors is essential for the design of approaches to combat infections.

DC-SIGN is a type II membrane protein with a C-type lectin extracellular domain^[12]. DC-SIGN plays an important role in establishing the initial contact between DCs and resting T lymphocytes through its recognition of ICAM-3, and mediates DC trafficking through interactions with endothelial ICAM-2^[13]. Therefore, DC-SIGN appears to be a critical mediator of the migratory cell and T cell-interacting capabilities. It has been identified that DC-SIGN helps HIV-1 to subvert DC functions and infect the host.

Recently, it was found that DC-SIGN is also an HCV envelope binding receptor and the HCV envelope glycoprotein E2 can bind to DC-SIGN through high-mannose N-glycans^[14]. It is becoming increasingly clear that other pathogens including cytomegalovirus (CMV), Ebola, SARS coronavirus, *Helicobacter pylori*, mycobacterium tuberculosis, *Leishmania*, *schistosoma mansoni*, etc, can subvert DC functions to escape immune surveillance^[15]. Therefore, investigation of the mechanisms of DC-mediated T lymphocyte priming, tolerance, effector cell migration and function may be helpful in restricting HCV dissemination and infection.

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Relationship between co-stimulatory molecule B7-H3 expression and gastric carcinoma histology and prognosis

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Abstract

AIM: To investigate the expression of co-stimulatory molecule B7-H3 in gastric carcinoma and adenoma tissue as well as normal gastric tissue and to explore the relationship between B7-H3 expression and pathological features and prognosis of gastric carcinoma.

METHODS: B7-H3 expression was detected in 102 samples of human gastric carcinoma and 10 samples of gastric adenoma and 10 samples of normal gastric tissue by immunohistochemical assay. Correlation between the expression of B7-H3 and the patients' age, sex, gastric carcinoma locus, tumor size, tissue type, tumor infiltration depth, differentiation degree, lymph node metastasis, and survival time was analyzed.

RESULTS: B7-H3 was expressed in all gastric adenoma samples and in 58.8% samples of gastric carcinoma. B7-H3 expression in gastric carcinoma samples was not related with the patients' age, sex, lymph node metastasis, and tumor size ($P > 0.05$), but with the survival time, infiltration depth of tumor and tissue type.

CONCLUSION: Detection of B7-H3 expression in gastric carcinoma tissue is beneficial to the judgment of the prognosis of gastric carcinoma patients and the choice of treatment.

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Key words: B7-H3 expression; Gastric carcinoma; Gastric adenoma; Prognosis

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INTRODUCTION

Tumor immune response is a very complicated physiological process involving many immune cells and molecules including membranous molecules and solubility factors. Studies indicate that a group of cell membrane molecules (co-stimulatory molecules) play a very important role in tumor immune response in the place of adjusted expression, interaction, and signal transmission. Co-stimulatory molecules are divided into two groups: TNF-TNF receptor superfamily and immunoglobulin superfamily. B7 family can transmit signals to co-stimulatory molecules of T cells^[1]. B7RP-1 (B7H, B7-H2), B7-H1 (PD-L1), B7-DC (PD-L2) and B7-H3 have been found and make it more complex to adjust the effects of this family^[2,3]. B7-H3 is a new co-stimulatory member of the B7 family and shares 20-27% identical amino acids with other members of the B7 family^[4]. It was reported that B7-H3 could stimulate CD4+ and CD8+T cells to increase the activity of CTL^[5]. B7-H3 is regarded as a positive regulation molecule. To our knowledge, expression of B7-H3 in gastric carcinoma has not been previously demonstrated.

MATERIALS AND METHODS

Materials

From 1998 to 1999, 102 samples were collected from gastric cancer patients who had undergone surgery in our hospital. The tumor was located in the mucosa in 10 cases, in shallow muscularis in 20 cases and in deep muscularis in 72 cases. The tumors were classified into well-, moderately- and poorly-differentiated adenocarcinomas. None of the patients received chemotherapy or radiotherapy before the surgery. We also investigated 10 specimens from gastric adenoma patients who had undergone surgery between 1998 and 1999.

The study was approved by the ethics committee of our hospital and all patients gave their written informed consent prior to enrolment.

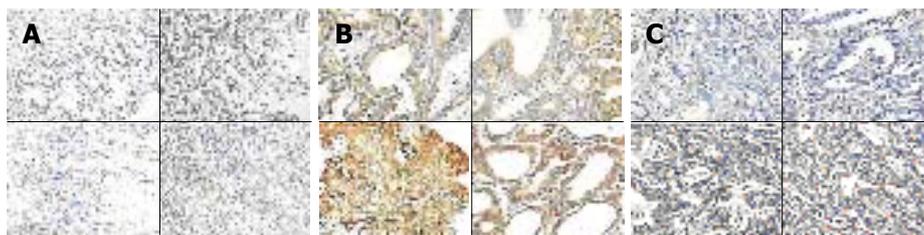


Figure 1 Staining of B7-H3 in normal gastric tissue (A), gastric adenoma tissue (B), and gastric carcinoma tissue (C).

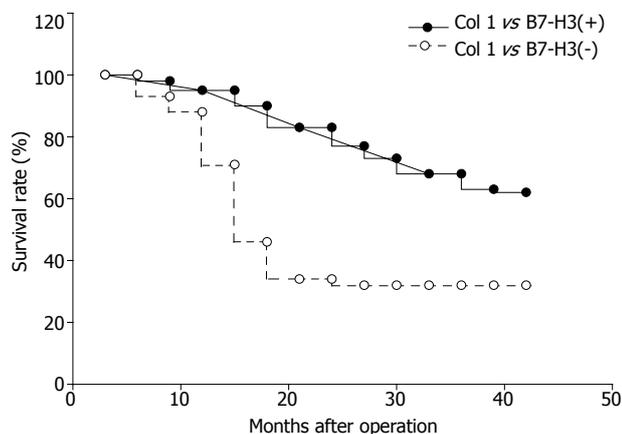


Figure 2 Correlation between B7-H3 expression and death of gastric carcinoma patients.

Immunohistochemical staining and assessment

Labeling was carried out with Elivision™ plus kit. The samples were fixed in formalin, embedded with paraffin wax and cut into 3- μ m-thick sections. The sections were dewaxed and rinsed and washed thrice with PBS (pH 7.4) for 3 min. The antigen of tissue was repaired and one drop or 50 μ L of 3% hydrogen peroxidase solution was added to each section and incubated at room temperature for 10 min to eliminate endogenous peroxidase activity. The sections were washed thrice with PBS for 3 min again. PBS was discarded; one drop or 50 μ L of primary antibody was added to each section and incubated at room temperature for 10 min or placed overnight at 40 °C. The sections were washed thrice with PBS for 5 min. PBS was discarded, one drop or 50 μ L of polymer accentuator (reagent A) was added to each section and incubated at room temperature for 20 min. After being washed thrice with PBS for 3 min each time, PBS was discarded and one drop or 50 μ L of enzyme-labeled anti-mouse/rabbit polymer (reagent B) was added to each section and incubated at room temperature for 30 min. The sections were washed thrice with PBS for 3 min each time. PBS was discarded; two drops or 100 μ L of freshly prepared DBA coloration fluid was added to each section and examined under microscope for 3-10 min. All the samples were fixed in formalin, embedded with paraffin wax and cut into 3- μ m-thick sections. The samples were assessed blindly by calculating the average ratio of positive cells in 10 vision fields (the plasma was stained brown-yellow) under a 400 \times microscope. If the average positive cell ratio was more than 20%, the sample was considered positive.

Statistical analysis

Difference between the groups was evaluated by χ^2 test

using SPSS version 10.0 for Windows and the relationship between the prognosis and various factors was evaluated by multivariable logistic regression. All *P* values were based on two-sided testing. *P* < 0.05 was considered statistically significant.

RESULTS

B7-H3 expression in gastric cancer and adenoma

B7-H3 was positively expressed in cell membrane and cytoplasm of gastric cancer and adenoma cells (Figure 1). B7-H3 was expressed in all the 10 specimens of gastric adenoma tissue and in 58.8% samples of gastric cancer tissue.

Relationship between B7-H3 expression and age, sex, and prognosis of patients

B7-H3 expression in gastric carcinoma tissue was not related with the patients' age and sex (*P* > 0.05, Table 1). The association of B7-H3 expression with the survival time after surgery indicated that B7-H3 expression was related to the prognosis of patients (Figure 2). The positive rate (74.5%) of B7-H3 expression was higher in gastric cancer patients who survived more than 5 years than in those who survived less than 2 years (43.1%, *P* < 0.01).

Relationship between B7-H3 expression and pathological features of gastric cancer

B7-H3 expression was not related with lymph node metastasis, tumor location and size (*P* > 0.05), but with survival time, infiltration depth of tumor and histology type (Table 1).

Related factors of gastric cancer patients' survival time

Univariate analysis suggested that the infiltration depth of tumor, survival time of patients and histology type of gastric cancer were related to B7-H3 expression. After adjustment for these factors, gastric cancer patients with high intratumor B7-H3 expression could survive 2-fold longer than those with low intratumor B7-H3 expression (risk ratio = 2.803; 95%CI = 1.051-7.477; *P* = 0.040), suggesting that B7-H3 expression might be an independent factor affecting the survival time of gastric cancer patients.

DISCUSSION

Human B7-H3 is also known as B7 relative protein 2 (B7RP-2) and its gene map on chromosome 15 is composed of seven exons and six introns. Mature B7-H3 protein has 316 amino acids and its molecular weight is 45-66 ku. It belongs to immunoglobulin superfamily and is a type I transmembrane protein composed of

Table 1 Correlation between tumor B7-H3 expression and pathologic features of gastric carcinoma patients

| Features | Total cases (n) | Positive cases (n) | Positive rate (%) |
|--|-----------------|--------------------|-------------------|
| Tumor location | | | |
| Top 1/3 layer of stomach | 31 | 19 | 61.2 |
| Middle 1/3 layer of stomach | 30 | 17 | 56.7 |
| Bottom 1/3 layer of stomach | 41 | 24 | 58.5 |
| Degree of differentiation | | | |
| Well-differentiated | 72 | 48 | 66.7 |
| Poorly-differentiated | 30 | 12 | 40.0 |
| Infiltration depth without | | | |
| Infiltration at deep muscular layer | 30 | 23 | 76.7 |
| With infiltration at deep muscular layer | 72 | 37 | 51.3 |
| Lymph node metastasis | | | |
| Negative | 54 | 33 | 61.1 |
| Positive | 48 | 27 | 56.3 |
| Survival time | | | |
| <2 years | 51 | 22 | 43.1 |
| >5 years | 51 | 38 | 74.5 |
| Primary tumor size | | | |
| <5 cm | 55 | 35 | 63.6 |
| >5 cm | 47 | 25 | 53.2 |

extracellular, transmembrane and intracellular regions. B7-H3 has been recently identified as a new co-stimulatory member of the B7 family and shares 20-27% identical amino acids with other members of the B7 family^[4]. It is extensively expressed in non-lymphoid tissues including the heart, liver, prostate, placenta, testis, pancreas, small, and large intestine and also in some tumor cell lines such as G361, HeLa S3, K562, A546, and SW480. B7-H3 was expressed in all the 10 specimens of gastric adenoma tissue and in 58.8% samples of gastric cancer tissue. We also found that B7-H3 was highly expressed in some epithelial tumor cell lines such as M435, A549, and H01299. A recent study found that B7-H3 can stimulate CD4+ and CD8+T cells to increase the activity of CTLs^[5]. In addition, B7-H3 increases the secretion of IFN- γ and can upregulate IL-8 and TNF- α . When B7-H3Ig is blocked by anti-B7-H3 multi-clone antibody, secretion of IFN- γ by DCs can be downregulated, indicating that B7-H3 is a positive regulatory molecule.

Our study suggested that B7-H3 expression in gastric carcinoma tissue was not related with the age and sex of patients, lymph node metastasis and size of tumor but with the survival time of patients, infiltration depth and histology type of tumor. The positive rate (74.5%) of B7-H3 expression was higher in gastric cancer patients who survived more than 5 years than in those who survived less than 2 years (43.1%), suggesting that B7-H3 expression is an independent factor affecting the survival time of gastric cancer patients and that B7-H3 might act as a positive regulatory factor in tumor immunology.

Sun *et al.*^[6] showed that intratumor injection of a mouse B7-H3 pcDNA3 expression plasmid leads to

complete regression of 50% tumors and can significantly inhibit tumor growth. Mice with their tumors completely regressed can resist a challenge with parental tumor cells. B7-H3-mediated anti-tumor immunity is mediated by CD8(+) T and NK cells, rather than CD4(+) T cells. These results indicate that B7-H3 interactions play a role in regulating cell-mediated immune responses against cancer.

However, Suh *et al.*^[7] found that B7-H3 inhibits immune response by inhibiting type 1 [T(H)1] responses and production of IFN- γ . A recent study showed that B7-H3 induces T cell proliferation and IFN- γ production through non co-stimulatory pathways^[8], indicating that B7-H3 may have more than one receptor on activated T cells.

Because the known receptor of B7-H3 has not been found, its function in the immune response is not clear. B7-H3 is extensively expressed in peripheral tissues, suggesting that it may play an important role in inflammation and transplantation immune response. B7-H3 is highly expressed in epithelial tumor cell lines and positively related to the prognosis of gastric cancer patients, indicating that B7-H3-deficient expression in tumor tissues may be closely associated with tumor immune escape.

In conclusion, B7-H3 is related to the development of gastric cancer and acts as an independent index for the diagnosis and prognosis of gastric cancer. Further study is needed to explore the exact relationship between B7-H3 and gastric cancer.

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RAPID COMMUNICATION

Clinical characteristics and prognostic factors of splenic abscess: A review of 67 cases in a single medical center of Taiwan

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Abstract

AIM: To analyze 67 cases of splenic abscess in a medical center of Taiwan during a period of 19 years.

METHODS: From January 1986 to December 2004, a total of 67 patients with splenic abscess were enrolled for the retrospective study. The clinical characteristics, underlying diseases, organism spectra, therapeutic methods, APACHE II scores, and mortality rates were analyzed.

RESULTS: There were 41 males and 26 females with the mean age of 54.1 ± 14.1 years. Multiple splenic abscesses (MSA) account for 28.4% and solitary splenic abscess in 71.6% of the patients. Twenty-six of sixty-seven patients (35.8%) had extrasplenic abscesses, with leading site of liver (34.6%). Microbiological cultures were positive in 58 patients (86.6%), with 71.8% in blood culture and 93.5% in abscess culture. Gram negative bacillus (GNB) infection predominated (55.2%), with leading pathogen of *Klebsiella pneumoniae* (22.4%), followed by gram positive coccus (GPC) infection (31%). Splenectomy was performed in 26 patients (38.8%), percutaneous drainage or aspiration in 21 (31.3%), and antibiotic therapy alone in 20 patients (29.9%). Eventually, 12 of 67 patients expired (17.9%). By statistics, spleen infected with GNB was likely to develop multiple abscesses compared with infection with GPC ($P=0.036$). Patients with GNB infection ($P=0.009$) and

multiple abscesses ($P=0.011$) experienced a higher mortality rate than patients with GPC infection and solitary abscess. The mean APACHE II score of 12 expired patients (16.3 ± 3.2) was significantly higher than that of the 55 survivals (7.2 ± 3.8) ($P < 0.001$).

CONCLUSION: MSA, GNB infection, and high APACHE II scores are poor prognostic factors. Early surgical intervention should be encouraged when these risk factors are present.

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Key words: Splenic abscess; Prognosis; Gram negative bacillus infection; APACHE II scores

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INTRODUCTION

Splenic abscess is a rare entity, with a reported frequency in autopsy series between 0.14% and 0.7%^[1,2,3]. It remains a subject of case reports and of small institutional series. Large case numbers reviewed in a single institute was rare and difficult. However, reviews from different series of geographic localizations and populations might be obscure on pathologic features in local area. Further, splenic abscess often occurs in the patients with underlying diseases^[4-8]. Patients with multiple splenic abscesses (MSA) or immunodeficiency are considered to have a poor prognosis and high mortality^[4,5,9]. Many surgeons had reported that splenectomy is a better way for treatment of splenic abscess^[3,5,8-10]. Recently, medical treatment and abscess drainage are proved to be efficient methods in the treatment of splenic abscess^[11-14]. However, various conditions interfere with the prognosis of splenic abscess, such as underlying diseases, abscess number and size, organism spectra, therapeutic methods and general conditions. There is no single risk factor that can predict

Table 1 Comparison of epidemiology and symptomatology in the reviews

| Variable | Chun <i>et al</i> ^[17] 1900-1977 | Nelken <i>et al</i> ^[5] 1977-1986 | Ooi <i>et al</i> ^[18] 1987-1995 | Present study 1986-2004 |
|-----------------------------|---|--|--|-------------------------|
| Number of cases | 173 | 189 | 287 | 67 |
| Male:female ratio(%) | 104:61 (63) | 125:64 (66) | 163:80 (67) | 41:26 (61) |
| Mean age (yr) | 36.8 | Not available | 41.1 | 54.1 |
| Age range (yr) | 6 mo-83 | 6 mo-82 | 6 mo-92 | 19-79 |
| Clinical presentations | | | | |
| Fever (%) | 95.4 | 84 | 90.8 | 85 |
| Left upper quadrant pain(%) | 42.1 | 39 | 49.8 | 43.3 |
| Splenomegaly(%) | 53.9 | 40 | 30.7 | 67.2 |
| Left pleural effusion(%) | 19.7 | Not available | 22.3 | 41.5 |

the prognosis exactly till now. APACHE II score is a method to evaluate the general condition and to easily get the score from the patient's general data^[15]. It has not been adopted in the evaluation of splenic abscess. To further elucidate the prognostic factors in splenic abscess, we have analyzed the various risk factors (including APACHE II score) of splenic abscess in a large series of 67 cases from a single medical center during a period of 19 years.

MATERIALS AND METHODS

From January 1985 to December 2004, a total of 67 patients with splenic abscess were enrolled. The diagnosis of splenic abscess was made if one of the following criteria was met: (1) microbiologically documented abscess (blood or splenic aspirate) with compatible splenic imaging studies of computed tomography (CT) or ultrasonography (US); (2) Pathologic microscopic examination of the spleen on autopsy, resection or aspirate that revealed abscess formation; (3) Operative findings of splenic abscess on exploratory laparotomy; (4) In the presence of typical clinical manifestations and finding of CT or US, the patients' conditions regressed after antibiotic therapy in cases without interventional therapies (only three cases in this study). Patients with diseases progressing to death and sterile culture were excluded from this study. The demographic, clinical, and laboratory characteristics, underlying diseases, organism spectra, abscess number and size, therapeutic methods, patients' APACHE II scores, and mortality rates were collected and analyzed. The size of abscess is recorded as the largest diameter in either solitary or multiple abscesses. The APACHE II score was evaluated following the criteria of Knaus' report^[15]. Age, chronic health problem score, Glasgow coma score, and 12 physiologic variables (vital signs, oxygenation, laboratory values; the most severely abnormal of each in the first 24 h of admission) were used to make a sum of scores^[15]. All the survival cases had been followed clinically for more than 6 mo. Comparison between groups of independent samples was assessed by the Student's *t*-test, one-way ANOVA (*post hoc* multiple comparisons by Scheffe's procedure). The associations between categorical variables were assessed using the χ^2 or Fisher's exact test. A two-tailed $P < 0.05$ was considered statistically significant.

RESULTS

There were 41 males and 26 females with the mean age of 54.1 ± 14.1 years (range 19-79 years). The mean age of males was 51.9 ± 13.6 years, and that of the females was 57.4 ± 14.5 years ($P = 0.123$). Sizes of abscess ranged from 1.5 to 16 cm, with a mean of 4.02 ± 2.56 cm.

The symptoms were fever in 57 patients (85.1%), left upper quadrant pain in 29 patients (43.3%), diffuse abdominal pain in 10 patients (14.9%), left chest wall pain in 3 patients (9%), and dyspnea in 5 patients (7.5%). Twenty five patients (37.3%) suffered from more than two symptoms at the time of diagnosis. The physical examination revealed splenomegaly in 34 patients (50.7%), left upper quadrant tenderness in 30 patients (44.7%), generalized abdominal tenderness in 9 patients (13.4%), left chest basilar rales in 6 patients (8.9%), and left chest basilar dullness in 5 patients (7.5%), leukocytosis over $10\,000/\text{mm}^3$ in 47 patients (70.1%), and leucopenia in 3 patients (4.5%) (Table 1).

Among the 67 patients, 54 of them (80.6%) had predisposing underlying diseases. Diabetes mellitus (DM) was the leading disease (25 patients, 46.3%). Seven of 54 patients (12.9%) had more than two underlying diseases simultaneously (Table 2). Statistical analysis revealed that the presence of underlying disease did not correlate with any clinicopathologic parameter, including size of abscess, numbers of abscess, age, gender, species of microorganism, and mortality rates of patients.

Of the images of splenic abscess, 65 patients underwent chest roentgenogram study. Forty of sixty-five patients (61.5%) had abnormalities as left lower lung infiltration (13 cases, 20%) or left pleural effusion (27 cases, 41.5%). Further, 66 of 67 patients underwent abdominal US and/or abdominal CT studies, in which 46 patients received both US and CT, 9 patients received US alone, and 11 patients received CT alone. One who had a solitary splenic abscess (SSA) had no image study because of emergent operation for peritonitis. The sensitivity of US and CT was 98.18% and 98.24%, respectively. Splenomegaly was found by images in 45 patients (67.2%). There were MSA in 19 cases (28.4%) and SSA in 48 cases (71.6%) (Table 3). Gas formation in the abscess was found in only 8 patients (11.9%), mainly in patients with gram negative bacillus (GNB) infections (7 cases). In addition

Table 2 Underlying and predisposing diseases in 67 cases of splenic abscess

| Underlying diseases (n = 67) | Solitary (SSA) (n = 48) | Multiple (MSA) (n = 19) | Extrasplenic abscess (n = 26) |
|---------------------------------|-------------------------------|-------------------------------|-------------------------------------|
| DM (20) | 17 | 3 | 10 ¹ |
| Endocarditis (7) | 7 | 0 | 4 ² |
| Pancreatitis (5) | 2 | 3 | 2 ³ |
| Pancreatitis+DM (2) | 1 | 1 | 0 |
| Pancreatic cancer (1) | 1 | 0 | 0 |
| Liver cirrhosis (2) | 1 | 1 | 1 ⁴ |
| Trauma (2) | 1 | 1 | 0 |
| MDS (2) | 0 | 2 | 2 ⁵ |
| SLE (2) | 2 | 0 | 0 |
| CHF (1) | 1 | 0 | 0 |
| Perforated peptic ulcer (1) | 1 | 0 | 0 |
| Colon perforation+ DM (1) | 1 | 0 | 1 ⁶ |
| Endocarditis+DM (1) | 1 | 0 | 0 |
| Biliary tract stone | 1 | 0 | 0 |
| AIDS (1) | 0 | 1 | 1 ⁷ |
| ALL (1) | 0 | 1 | 0 |
| Aplastic anemia (1) | 1 | 0 | 0 |
| ESRD+ovarian cancer (1) | 0 | 1 | 0 |
| COPD+CHF (1) | 1 | 0 | 0 |
| Lung cancer+DM (1) | 1 | 0 | 0 |
| Unknown (13) | 8 | 5 | 5 ⁸ |

DM, diabetes mellitus; MDS, myelodysplastic syndrome; SLE, systemic lupus erythematosus; CHF, congestive heart failure; AIDS, acquired immunodeficiency syndrome; ALL, acute lymphocytic leukemia; ESRD, end stage renal disease; COPD, chronic obstructive pulmonary disease. ¹Liver: 3; subphrenic area: 2; pancreas: 1; anus: 1; gluteal muscle: 1; retroperitoneal cavity: 1; brain+lung: 1. ²brain: 2; aortic valve: 1; lung: 1. ³pancreas: 2. ⁴gluteal muscle: 1. ⁵liver: 2. ⁶subphrenic area: 1. ⁷liver: 1. ⁸liver: 2; brain+lung: 1; retroperitoneal cavity: 1; subphrenic area: 1.

to spleen, 26 of 67 patients (35.8%) had extrasplenic abscesses, with leading site of liver (Table 4). Among them two patients showed two sites of extrasplenic abscesses in the lung and brain. By statistics, we found that MSA tended to develop in non-diabetic patients ($P=0.053$), and in abscess with GNB infection ($P=0.036$, Tables 2 and 3). Furthermore, MSA patients experienced a higher mortality rate than SSA patients ($P=0.011$, Table 5). Seven of nineteen (36.8%) MSA patients, in contrast to 5 of 48 (10.4%) SSA patients expired eventually. *Klebsiella pneumoniae* (*K. pneumoniae*) (70%) and staphylococcus aureus (60%) were most common pathogens to develop extrasplenic abscess (Table 3). However, statistical analysis revealed no significant correlation between the presence of extrasplenic abscess and any clinicopathologic factor.

Bacteriologic studies revealed that positive microbiological cultures were found in 58 patients (86.6%), and sterile in the other 9 patients (Table 3). Blood culture was positive in 46 of 64 patients (71.8%), and abscess culture in 43 of 46 patients (93.5%). Twenty-eight of thirty patients who had both positive blood and abscess cultures revealed identical pathogens. The microorganisms of 58 cases of positive culture were predominantly GNB (32 cases, 55.2%), with *K. pneumoniae* as the leading pathogen

Table 3 Microorganisms of blood culture and abscess culture of splenic abscess

| Microorganism (n = 67) | Solitary (n = 48) | Multiple (n = 19) | Extrasplenic abscess (n = 26) |
|--|----------------------|----------------------|----------------------------------|
| Aerobes | | | |
| Gram positive-18 | | | |
| <i>Streptococcus viridans</i> (SV) (8) | 7 | 1 | 2 ¹ |
| <i>Staphylococcus aureus</i> (5) | 5 | 0 | 3 ² |
| <i>Enterococcus species</i> (5) | 5 | 0 | 1 ³ |
| Gram negative-32 | | | |
| <i>Escherichia coli</i> (11) | 8 | 3 | 4 ⁴ |
| <i>Klebsiella pneumoniae</i> (KP)(10) | 4 | 6 | 7 ⁵ |
| <i>Pseudomonas species</i> (PS) (5) | 4 | 1 | 1 ⁶ |
| <i>Salmonella species</i> (5) | 4 | 1 | 2 ⁷ |
| <i>Proteus</i> (1) | 1 | 0 | 0 |
| Gram positive+gram negative-4 | | | |
| SV+KP (2) | 2 | 0 | 1 ⁸ |
| SV+PS (1) | 0 | 1 | 1 ⁹ |
| SV+PS+KP (1) | 0 | 1 | 0 |
| Anaerobes-2 | | | |
| Gram positive | | | |
| <i>Propionibacterium acnes</i> (1) | 1 | 0 | 0 |
| Gram negative | | | |
| <i>Bacteroides fragilis</i> (1) | 1 | 0 | 1 ¹⁰ |
| Mycobacterium-1 | | | |
| Tuberculosis (1) | 0 | 1 | 1 ¹¹ |
| Fungus-1 | | | |
| <i>Cryptococcus neoformans</i> (1) | 0 | 1 | 1 ¹² |
| Sterile culture-9 | 6 | 3 | 1 ¹³ |

¹Brain: 1; liver: 1. ²lung: 1; retroperitoneum: 1; brain: 1. ³retroperitoneum: 1. ⁴liver: 1; subphrenic area: 1; gluteal muscle: 1; pancreas: 1. ⁵liver: 4; brain+lung: 2; subphrenic area: 1. ⁶anus: 1. ⁷aortic valve: 1; subphrenic area: 1. ⁸subphrenic area: 1. ⁹gluteal muscle (1). ¹⁰pancreas: 1. ¹¹liver: 1. ¹²liver: 1. ¹³pancreas: 1.

(22.4%, Table 3). Statistical analysis revealed that abscesses with GNB were likely to develop multiple focus compared with abscesses with gram positive coccus (GPC) ($P=0.036$). There were 11 MSA in 32 patients (34.4%) with GNB. In contrast, only one of the 18 patients (5.6%) with GPC had MSA. Furthermore, patients with GNB infection also experienced a higher mortality rate than patients with GPC ($P=0.009$). Ten of 32 GNB (31.2%) patients, in contrast to none of 18 (0%) GPC patients expired eventually (Table 5).

In the treatment of splenic abscess, all patients received antibiotic therapy. Splenectomy was performed in 26 patients (38.8%), percutaneous drainage or aspiration in 21 (31.3%), and antibiotic therapy alone in 20 patients (29.9%). Eventually, 12 patients expired (17.9%) at the end of follow-up. Three patients with percutaneous drainage or aspiration initially were finally referred for splenectomy, and all of them survived. Two mortality patients who underwent splenectomy died of predisposing factors as extensive pancreatitis and severe abdominal trauma rather than splenic abscess itself. The mortality rate was 7.7% in patients treated with splenectomy, 28.6% in patients with percutaneous drainage or aspiration, and 20% in patients with antibiotic therapy alone. There was no difference of

Table 4 Locations of extrasplenic abscesses

| Location | Case number (%) |
|------------------------|-----------------------|
| Liver | 9 (34.6) |
| Subphrenic area | 4 (15.4) |
| Pancreas | 3 (11.5) |
| Brain | 3 (11.5) ¹ |
| Lung | 3 (11.5) ¹ |
| Retroperitoneal cavity | 2 (7.7) |
| Gluteal muscle | 2 (7.7) |
| Aortic valve | 1 (3.85) |
| Anus | 1 (3.85) |

¹Twenty-six patients had single, and two patients had two sites of extrasplenic abscesses (lung+brain).

mortality rate between the groups (Table 5). But marginally better outcome was found in patients with splenectomy than patients with percutaneous drainage or aspiration ($P=0.06$).

For analysis of patients' outcome, we first applied the APACHE II score for further correlation study. The twelve expired patients had high APACHE II scores over 15 except one. The mean score of 12 expired patients (16.1 ± 3.2) was significantly higher than that of the 55 survivals (7.3 ± 3.8) ($P < 0.001$). It was also found that higher scores presented in fatal cases of each category, regardless of underlying disease, abscess number and size, organism spectrum, and different therapeutic methods (data not shown). To summarize our results, we found that patients with multiple splenic abscesses, GNB infection had a higher mortality rate than patients with solitary splenic abscess and GPC infection. Mortality patients also had a higher mean APACHE II score than survival patients (Table 5).

DISCUSSION

Reports on splenic abscess between 1900 and 1977^[16], between 1977 and 1986^[4], and between 1987 and 1995^[17] showed a great variety of causative pathogens with a wide range of demographic and clinical conditions. Reviews of large series of patients with splenic abscess are capable of shedding light on the pathogenesis and clinical characteristics of splenic abscess^[4,16,17]. Reviews from different geographic localizations and populations might obscure some specific pathologic features in the local area. Therefore, our large series review in single medical center might provide a further insight of the pathogenesis of splenic abscess in Taiwan.

Our patients experienced an older age, and higher percentage of splenomegaly and more left lung change (infiltration or pleural effusion) than that reported by other studies^[4,16,17] (Table 1). These might be attributed to extensive studies by images. US and CT may help to detect small amount of pleural effusion which is subtle in CXR. Further, US and CT are also more sensitive to demonstrate splenomegaly than physical examination did. With a reported sensitivity of 96%, CT was considered to be superior to US (sensitivity 75-90%) for detecting splenic

Table 5 Prognostic factors of splenic abscess

| Variables | Category (n) | Outcome | | P |
|------------------------------|-------------------|------------------|-----------------------|---------------------|
| | | Cure (n = 55, %) | Mortality (n = 12, %) | |
| Gender | Female (26) | 21 (81) | 5 (19) | NS ¹ |
| | Male (41) | 34 (83) | 7 (17) | |
| Abscess number* | Solitary (48) | 43 (89) | 5 (11) | 0.011 ¹ |
| | Multiple (19) | 12 (63) | 7 (37) | |
| Extrasplenic abscesses | Without (43) | 36 (84) | 7 (16) | NS ¹ |
| | With (24) | 19 (79) | 5 (21) | |
| Underlying diseases | Without (15) | 12 (80) | 3 (20) | NS ¹ |
| | With (52) | 43 (83) | 9 (17) | |
| Diabetes | Without (41) | 33 (80) | 8 (20) | NS ¹ |
| | With (26) | 22 (85) | 4 (15) | |
| Microorganism ^a | GNB (32) | 22 (69) | 10 (31) | 0.0091 ^a |
| | GPC (18) | 18 (100) | 0 (0) | |
| | GNB+GPC (4) | 4 (100) | 0 (0) | |
| | Others (4) | 2 (50) | 2 (50) | |
| | Sterile (9) | 9 (100) | 0 (0) | |
| Treatment | Splenectomy (26) | 24 (92) | 2 (8) | NS ¹ |
| | PD or FNA+AT (21) | 15 (71) | 6 (29) | |
| | AT alone (20) | 16 (80) | 4 (20) | |
| Age | | 53.3±13.4 | 57.3±17.1 | NS ² |
| Size of abscess ² | | 5.1±3.0 | 4.0±2.6 | NS ² |
| Leukocytes/mm ³ | | 13.1±4.5 | 9.6±5.9 | NS ² |
| APACHE II score* | | 7.3±3.8 | 16.1±3.2 | <0.001 ² |

GNB, gram negative bacillus; GPC, gram positive cocci; PD, percutaneous drainage; FNA, fine needle aspiration; AT, antimicrobial therapy. Categorical data were compared by: 1: χ^2 test or *F*-test, 2: *t*-test. ^a $P < 0.05$ GNB vs GPC. NS: not significant.

abscess^[12,17]. However, we found that the sensitivity of both CT and US in this study was equal and extremely high (98%). Therefore, US is an easy diagnostic and therapeutic tool for splenic abscess^[18].

The cause of splenic infection has been most often a metastatic infection or contiguous distant infection^[4,16,17]. Recently, changing lifestyles resulted in an increasing prevalence of DM, malignancies, and immunosuppression becomes advanced therapeutic methods. These conditions constitute the increasing predisposing risk for the development of splenic abscess. Underlying diseases such as DM, endocarditis, and other diseases were reported not to be a good predictor for prognosis before. However, immunodeficiency is considered to be a poor prognostic factor and caused a high mortality^[7-8]. In our series, the most common predisposing factors were still metastatic or contiguous infections. But 10 patients had immunodeficiency disorders (acquired immunodeficiency disease, myelodysplastic syndrome, systemic lupus erythematosus, acute lymphocytic leukemia, malignancies after chemotherapy, and aplastic anemia), and six of ten patients suffered from MSA and expired. Based on the limited case numbers, we cannot arrive at any conclusion about the prognostic role of immunodeficiency disorder.

But actually, we have found many mortality patients with severe illness (due to immunodeficiency) during the initial patients search for this study. They were excluded from this study mostly because of progression of disease to death with uncertain microbiological information. In these patients, multiple microabscesses were hard to be differentiated from micrometastatic or infarction lesions. Therefore, the real incidence of splenic abscess and the percentages of immunodeficient risk might be underestimated.

In contrast to previous reports (*Streptococcus* and *Staphylococcus* species predominant)^[3,4,7,8,10], our series revealed GNB were the leading pathogens causing splenic abscess (55.2%). Among GNB, *K. pneumoniae* was the most frequently encountered pathogen (22.4%). It has been reported to be the most common pathogen of liver abscess in Taiwan^[19]. Further, metastatic splenic infections from liver abscesses caused by *K. pneumoniae* were rare^[19]. Primary splenic abscess due to *K. pneumoniae* has also been rarely addressed before. In the present study, we have found that extrasplenic abscesses existed in 26 of 67 patients (38.8%), with leading site of liver in 9 (34.6%). Among the 9 liver abscesses, *K. pneumoniae* accounted for 44% of pathogens. We wonder whether it was a sequela of metastatic infection from liver to spleen or a co-infection of both. Nevertheless, these results indicated the significant role of GNB infection in splenic abscess. By statistics, we found that patients with GNB infection were prone to develop multiple splenic abscess ($P=0.036$) and had a higher mortality rate ($P=0.009$) than patients with GPC did. It is a novel finding which was never reported.

In this series, the treatment was carried out by antibiotic therapy alone, percutaneous splenic aspiration and/or drainage, or surgical methods. The mortality rate in patients receiving antibiotic therapy alone or percutaneous splenic drainage was marginally higher than that undergoing splenectomy. This result came from (1) the patient number in each different treatment group was still small, and (2) surgical treatment was not performed in some patients, if patient's condition was too ill to tolerate an operation. Based on these, the prognosis of our patients cannot be predicted by treatment methods exactly. In fact, even the previous inferences were mostly derived from comparison of patients in different series because of the rarity of disease^[5,16,17]. There is a lack of a prospective cohort study to demonstrate which therapy is superior to others. To overcome this, we first adopted APACHE II score to predict the patients' outcome. The fatal patients had higher APACHE II score (16.1 ± 3.2) than the survivals (7.3 ± 3.8) ($P < 0.001$). It was also found that higher scores presented in fatal cases of each category, regardless of underlying disease, abscess number and size, organism spectrum, and different therapeutic methods. Among the nine expired patients, eight had a score of over 15. Therefore, we thought that a score over 15 was a risk point for splenic abscess, the sensitivity, specificity, positive

predict value, and negative predict value would be 89%, 94%, 80%, and 97%, respectively.

In conclusion, we have obtained a novel finding of GNB serving as a leading pathogen in splenic abscess and provided evidence that multiple splenic abscesses, GNB infection, and high APACHE II scores are poor prognostic factors. Aggressive and early surgical intervention of splenic abscess should be encouraged when these risk factors are present.

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Telomerase activity and human telomerase reverse transcriptase expression in colorectal carcinoma

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expression of telomerase activity and hTERT, $P=0.021$).

CONCLUSION: Telomerase activity is closely correlated with the occurrence, development and metastasis of colorectal carcinoma. Overexpression of hTERT may play a critical role in the regulation of telomerase activity.

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Key words: Colorectal carcinoma; Telomerase activity; hTERT expression

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Abstract

AIM: To study the activity of telomerase and the expression of human telomerase reverse transcriptase (hTERT) in colorectal carcinoma and its adjacent tissues, normal mucosa and adenomatoid polyp, and to evaluate their relation with carcinogenesis and progression of colorectal carcinoma.

METHODS: Telomerase activity and hTERT expression were determined in 30 samples of colorectal carcinoma and its adjacent tissues, normal mucosa and 20 samples of adenomatoid polyp by modified telomeric repeat amplification protocol (TRAP), enzyme-linked immunosorbent assay (ELISA) and immunohistochemical method.

RESULTS: Telomerase activity and hTERT expression were 83.33% (25/30) and 76.67% (23/30) respectively in colorectal carcinoma, which were obviously higher than those in paracancerous tissues (13.33%, 16.67%), normal mucosa (3.33%, 3.33%) and adenomatoid polyp (10%, 10%). There was a significant difference between colorectal carcinoma and other tissues ($P=0.027$). The telomerase activity and hTERT expression were higher in colorectal carcinoma with lymphatic metastasis than in that without lymphatic metastasis ($P=0.034$). When the histological classification and clinical stage were greater, the telomerase activity and hTERT expression increased, but there was no significant difference between them. In colorectal carcinoma, the telomerase activity was correlated with hTERT expression (positive vs negative

INTRODUCTION

Colorectal carcinoma is one of the most common malignant tumors in digestive system, threatening human life and health. Recent investigations demonstrated that the telomerase activity is significantly increased in human malignant tumors, but is not expressed in normal somatic cells^[1,2], suggesting there is a close relation between telomerase activity and malignant tumors. In order to study the role of telomerase activity in the carcinogenesis and progression of colorectal carcinoma as well as the correlation between telomerase activity and hTERT expression, the telomerase activity and hTERT expression were determined in colorectal carcinoma and its adjacent tissues, normal mucosa and adenomatoid polyp in this study.

MATERIALS AND METHODS

Patients and specimens

Thirty specimens of colorectal carcinoma and corresponding paracancerous tissues and normal mucosa were obtained by surgical resection, 20 specimens of adenomatoid polyp were taken during endoscopic examination. All patients were diagnosed pathologically and no patient received radiotherapy or chemotherapy before the sampling. Clinical staging showed Dukes' A in none, Dukes' B in 2 cases, Dukes' C in 24 cases, and Dukes' D in 4 cases. Well-differentiated adenocarcinoma

Table 1 Telomerase activity and hTERT expression in four different groups

| Group | n | Telomerase n (%) | hTERT n (%) |
|------------------------------|----|-------------------------|-------------------------|
| Colorectal carcinoma | 30 | 25 (83.33) ^a | 23 (76.67) ^a |
| Adjacent peritumoral tissues | 30 | 4 (13.33) | 5 (16.67) |
| Adenomatoid polyp | 20 | 2 (10.00) | 2 (10.00) |
| Normal mucosa | 30 | 1 (3.33) | 1 (3.33) |

^a $P < 0.05$ ($\chi^2 = 59.58$, $\chi^2 = 49.23$) vs adjacent peritumoral tissues, adenomatoid polyp and normal mucosa.

Table 2 Relation between telomerase activity and hTERT expression and clinical pathologic factors of colorectal carcinoma

| Group | n | Telomerase (%) | hTERT (%) |
|------------------------------|----|---------------------------|---------------------------|
| Dukes' A | 0 | 0 | 0 |
| Dukes' B | 2 | 1/2 ^a | 1/2 ^a |
| Dukes' C | 24 | 83.33 (20/24) | 83.33 (20/24) |
| Dukes' D | 4 | 4/4 ^a | 2/4 ^a |
| High differentiation | 5 | 2/5 ^a | 3/5 ^a |
| Moderate differentiation | 8 | 6/8 ^a | 5/8 ^a |
| Poor differentiation | 17 | 100.00 (17/17) | 88.24 (15/17) |
| With lymphatic metastasis | 10 | 80.00 (8/10) ^b | 70.00 (7/10) ^b |
| Without lymphatic metastasis | 20 | 0.00 (0/20) | 5.00 (1/20) |

^aindicates cases less than 10 not included in the percentage; ^b $P < 0.01$ vs the group without lymphatic metastasis

was found in 5 cases, moderately-differentiated adenocarcinoma in 8 cases and poorly-differentiated adenocarcinoma in 17 cases. Ten patients had lymph node involvement and 20 had no lymph node involvement. Of the patients with adenomatoid polyp, two had accompanying moderate-severe atypical hyperplasia. Specimens were collected within 30 minutes *in vitro*. Each specimen was divided into two parts, one for pathological diagnosis and immunohistochemical staining and the other for telomerase activity assay.

TRAP PCR ELISA protocol for telomerase activity assay

Telomerase activity was detected with a kit (Roche, Germany). Primers TS (5' AATCCGTCGAGCAGAGTT) and CX (5' CCCTTACCCTTACCCTTACCCTAA) were designed. Thirty cycles of PCR were performed at 25 °C for 30 min, at 94 °C for 5 min, at 94 °C for 30s, at 50 °C for 30s, at 72 °C for 90s, and a final extension at 72 °C for 10 min. The PCR products were analyzed and defined positive when $A > 0.2$ on the reading of the microplate reader.

hTERT expression detection by immunohistochemical staining

Expression of hTERT was detected by immunohistochemical assay with the kit provided by Beijing Zhongshan Golden Biotechnology Co. Ltd according to the manufacturer's instructions. S-P method was adopted and positive calls were calculated and compared according to the accessory tester of the product. A negative control was prepared for each sample using

Table 3 Correlation between telomerase activity and hTERT expression

| Telomerase | hTERT | | | P |
|------------|-------|----|---|-------|
| | n | + | - | |
| + | 25 | 20 | 5 | <0.05 |
| -- | 5 | 3 | 2 | |

PBS as the primary antibody. Microscopically, no staining was negative, karyon and perikaryon cytoplasm with brown granules were defined as positive cells.

Statistical analysis

Statistical analyses were carried out with PEMS statistical-software. Chi-square test or Fisher's exact test was used for data processing. $P < 0.05$ was considered statistically significant.

RESULTS

Telomerase activity and hTERT expression in colorectal carcinoma and its adjacent tissues, normal mucosa and adenomatoid polyp

The high telomerase activity and hTERT expression were found in 25 (83.33%) and 23 (76.67%) out of the 30 specimens of colorectal carcinoma, but in only 4 (13.33%) and 5 (16.67%) specimens of adjacent peritumor tissues, in 1 (3.33%) and 1 (3.33%) specimens of normal mucosa, in 2 (10.00%) and 2 (10.00%) out of the 20 specimens of adenomatoid polyp (Table 1).

Relation between telomerase activities and hTERT expression and clinical pathologic factors of colorectal carcinoma

The telomerase activity and hTERT expression had a close relation with lymphatic metastasis, and the positive expression rate in the patients with lymphatic metastasis was significantly higher than that in the patients without lymphatic metastasis ($P < 0.05$). The telomerase activity and hTERT expression increased when the histological grade and clinical staging were greater, but the difference was of no statistical significance ($P > 0.05$, Table 2).

Relation between telomerase activity and hTERT expression

The expression coincidence rate of telomerase activity and hTERT expression in colorectal carcinoma and its adjacent tissues, normal mucosa and adenomatoid polyp was 92% (23/25), 80% (4/5), 100% (1/1), 100% (2/2) respectively. The total coincidence rate of telomerase activity and hTERT expression was 90.91% (30/33). Of the 30 cases of colorectal carcinoma, 20 cases had positive expression in telomerase activity and hTERT expression, 2 showed no telomerase activity and hTERT expression. The conformity rate was 73.33% (22/30) ($P < 0.05$, Table 3).

DISCUSSION

Telomerase is a ribonucleoprotein complex (a cellular

reverse transcriptase) consisting of three components: human telomerase RNA (hTR), telomerase-associated protein 1 (TP1/TLP) and human telomerase reverse transcriptase (hTERT). The first two components are expressed constitutively in both normal and tumor tissues and their expression levels are not correlated with the telomerase activity, whereas hTERT expression is closely correlated with telomerase activity in cells and tissues. Telomerase uses its internal RNA component as a template to synthesize telomeric DNA (TTAGGG)_n, participating in the maintenance of telomere length and immortalization of cells^[3]. Shay *et al*^[4] reported that the total positive rate of telomerase activity was 85% in more than 20 malignant tumors and merely 9% in adjacent peritumor tissues and normal tissues, suggesting that telomerase activity is closely associated with malignancies. Our study showed that the telomerase activity and hTERT expression in colorectal carcinoma (83.33% and 76.67% respectively) were much higher than those in adjacent peritumoral tissues, normal mucosa and adenomatoid polyp. The telomerase activity and hTERT expression were positive in 2 cases of adenomatoid polyp with a diameter >2 cm, suggesting that telomerase activity may be an important index of canceration. This result is consistent with the findings of Tang *et al*^[5]. The telomerase activity was higher in colorectal carcinoma with lymphatic metastasis than in that without lymphatic metastasis ($P < 0.01$), indicating that the level of telomerase activity is closely related with lymphatic metastasis. The increased telomerase activity was in accord with the histological grade and staging of tumor, suggesting that telomerase activity plays a key role in the occurrence and development of colorectal carcinoma, and can be used as a marker for the early diagnosis and prognostic estimation of colorectal carcinoma.

hTERT is a telomerase reverse transcriptase isoform which is highly expressed in cell lines of positive telomerase^[4]. hTERT transcription translated with hTR is indicative of telomerase activity *in vitro*. The activity of telomerase decreases or even disappears if the amino acid in hTERT is changed, whereas introduction of hTERT into normal cells activates the telomerase and prolongs cell life span while the karyotype and phenotype of cells remain normal^[6-9]. In our study, the expression of hTERT was closely associated with telomerase activity, the coincidence rate of telomerase activity and hTERT

expression was as high as 90.91% (30/33), suggesting that telomerase activity is related with hTERT expression. The results support the opinion that hTERT is an important rate-limiting determinant of telomerase activity and the expression level of hTERT is directly associated with the telomerase activity. Since hTERT test can be conducted in paraffin-embedded tissues, more samples are available for telomerase activity test and hTERT may be used as a new tumor marker.

In conclusion, telomerase activity and hTERT expression in colorectal carcinoma are closely related. hTERT may play an important role both in the activation of telomerase and in the formation and development of colorectal carcinoma. hTERT can be used as a new marker for the early diagnosis of colorectal carcinoma.

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RAPID COMMUNICATION

***Helicobacter pylori* infection in the pharynx of patients with chronic pharyngitis detected with TDI-FP and modified Giemsa stain**

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pharynx, which was remarkably higher ($P=0.042$) than that in the patients without stomach ailment history (1 case, which was 2.9%).

CONCLUSION: *H pylori* may not be detected in the pharynx of healthy people. Chronic pharyngitis may be related to *H pylori* infection. The infection rate with *H pylori* in the pharynx is higher in patients with stomach ailment histories than in patients without stomach ailment histories, suggesting that chronic pharyngitis may be related to stomach ailment history.

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Key words: Chronic pharyngitis; *H pylori*; Modified Giemsa stain

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Abstract

AIM: To detect whether there is *Helicobacter pylori* (*H pylori*) colonization in the pharynx mucous membrane of healthy people and whether chronic pharyngitis is related to *H pylori* infection.

METHODS: Fifty cases of chronic pharyngitis refractory over three months were prospectively studied from March 2004 to August 2004 in the otolaryngology outpatient department of the Second Hospital of Xi'an Jiaotong University. Template-directed dye-terminator incorporated with fluorescence polarization detection (TDI-FP) and modified Giemsa stain were used to examine pharynx mucous membrane tissue for *H pylori* colonization in the patients with chronic pharyngitis and the healthy people as a control group.

RESULTS: In the control group, no people were detected to have *H pylori* in the pharynx. In contrast, in 50 cases with chronic pharyngitis, 19 (38.0%) cases were *H pylori* positive with a TDI-FP assay and 4 (8%) cases were TDI-FP positive with Giemsa staining in the pharynx. Sixteen of the 50 pharyngitis cases had stomach ailment history, 11 cases (68.8%) of these 16 patients were determined to be *H pylori* positive in the pharynx with the TDI-FP assay. χ^2 test showed that this infection rate was remarkably higher ($P=0.0007$) than that in the cases without stomach ailment history. Giemsa staining showed that 3 cases (18.8%) of the patients with stomach ailment history were infected with *H pylori* in the

INTRODUCTION

Chronic pharyngitis is a common disease in the otolaryngology clinic. Pharyngitis, bronchitis, and pneumonia represent the most common respiratory tract infections. Upper respiratory tract infections are common and important. These include sinusitis, otitis media, and pharyngitis/tonsillitis. Many patients visiting an office-based physician report "sore throat, foreign-body sensation in the throat" as their primary reason for the visit^[1]. Acute pharyngitis may be caused by a wide variety of microbial agents, but some of the most common and potentially dangerous microorganisms are group A beta-hemolytic streptococci (GABHS). For example, streptococcal pharyngitis is responsible for about 5% to 17% of sore throats in adults. However, the causative microorganisms in many cases remain unclear^[2]. An important risk factor for chronic pharyngitis is gastroesophageal reflux disease (GERD). GERD is a common disorder in the Western population and is also common in Xi'an's adult population. The etiology and pathogenesis of GERD are probably associated with other conditions that are also risk factors

for chronic pharyngitis, including functional dyspepsia (FD), irritable bowel syndrome (IBS), and some respiratory and laryngopharyngeal diseases^[3].

The Gram-negative bacterium *Helicobacter pylori* (*H pylori*) is associated with severe gastric pathologies, including peptic ulcers, chronic active gastritis and gastric cancer. This microorganism is able to invade and colonize in the human stomach, gastric juice, saliva, and faeces of patients^[4-6]. Moreover, as relates to the current study, investigations using the CLO (Campylobacter-like organism) test and PCR showed that there was a high rate of *H pylori* colonization in tonsil and adenoid tissues^[7,8], however, another investigation indicated the opposite result^[9]. Therefore, we sought to detect whether there was *H pylori* colonization in the pharynx in healthy people, as compared to patients suffering from chronic pharyngitis. We used two methods to detect *H pylori* infection in the pharynx: template-directed dye-terminator incorporated with fluorescence polarization detection (TDI-FP) and modified Giemsa stain. From this study, it can be determined more definitively whether there is a relationship between *H pylori* infection of the pharynx and chronic pharyngitis; moreover, it can be determined if a history of certain gastric diseases may contribute to this infection.

MATERIALS AND METHODS

Patients

Two groups were studied, a group with chronic pharyngitis and a control group without chronic pharyngitis. The group with chronic pharyngitis consisted of 50 cases of chronic pharyngitis refractory over three months that were studied from March, 2004 to August, 2004 in the otolaryngology outpatient department. Among them, 32 cases were females and 18 cases were males, with ages ranging from 23 to 52 years old. The shortest disease history was 3 mo and the longest was 8 years. Major symptoms included foreign body sensation, dry sensation, angina in the pharynx, nausea, cough and belching. Clinical examination showed congestion in the pharynx mucosa, lymph follicle hyperplasia, and slightly swollen tonsils. Of these 50 cases, there were 34 cases with no specific stomach ailment history and 16 cases with gastric ulcers or chronic active gastritis. There were 20 cases in the control group. Of these, 12 cases were females and 8 cases were males. Ages ranged from 20-51 years old. The people in the control group had no specific pharyngitis or stomach ailment history, such as gastric ulcer or chronic active gastritis. In addition, all the people in the above two groups (1) had no other system diseases; (2) had no antibiotics treatment 10 d before examination; and (3) had no clear history of metronidazole, amoxicillin or tetracycline treatment. To collect tissue from the pharynx of each patient, the surface of the pharynx mucous membrane was sprayed with 20 g/L amethocaine for anaesthesia. Epithelial tissue in the pharynx was then collected with a sterilized curette. The protocol was approved by the Institutional Human Subject Committee at Xi'an Jiaotong University.

Methods

The biopsied epithelial tissue from the pharynx of each patient was routinely fixed in 4 g/L formaldehyde and embedded in paraffin wax. Histological sections were stained with routine modified Giemsa.

Fluorescence Polarization-Capable Instrument-Victor, AmpliTaq DNA Polymerase, shrimp alkaline phosphatase, *E. coli* exonuclease I, mixtures of TAMRA-ddTTP and RP110-ddGTP were purchased from PerkinElmer. The pGEM-T-Easy Vector System TA Cloning Kit was purchased from Promega. The ABI 377 and BigDye Terminator Cycle Sequencing Kit were purchased from Applied Biosystem(s). All reactions were run and read in 96-well black-skirted plates purchased from MJ Research. The specific probes and terminators of *H pylori* were designed by DNA Star based on a specific target sequence. All probes were synthesized by Sbsbio (Beijing, China).

Scrapes or biopsies were collected and washed into 5 mL PBS (pH 7.2). In each case, the cell suspension was centrifuged for 5 min at 10 000 r/min. The cell pellet was re-suspended and mixed in 1.5 mL TE buffer (200 mg/L proteinase K, 10 mmol/L Tris, 0.1 mmol/L EDTA, 1 g/L SDS, 3 g/L Triton X-100) at 37°C for 12 h. The suspension was incubated at 95°C for 10 min to inactivate proteinase K, and then it was centrifuged for 5 min at 10 000 r/min. The supernatant DNA was used as a template for PCR.

Amplification of *H pylori* DNA by using a conservative primer pair

For extensive detection of *H pylori* DNA, each sample DNA was first amplified in a 25 µL reaction mixture containing 1 µL of DNA extract, 2 µL of 6.25 pmol/L common conservative primer pair (P1, 5' tgcccgccttcactaacccca 3'; P2, 5' gtcagccactttgccacttctacag 3', *H pylori* urease B (*ureB*) gene (gene bank accession number AY295085), 10×PCR reaction buffer (10 mmol/L Tris-HCl, 50 mmol/L KCl), 2.5 µL of 2.5 mmol/L dNTP, 1.5 µL of 25 mmol/L MgCl₂, and 2.5 µL of 0.25 U AmpliTaq DNA Polymerase. The mixture was denatured initially for 5 min at 94°C, followed by 30 cycles of amplification in a PCR processor. Each cycle included a denaturing step of 94°C for 1 min, an annealing step of 45°C for 1 min and a chain elongation step of 72°C for 1.5 min. The final elongation step was then prolonged for another 5 min. The size of all *H pylori* genotyping PCR products was predicted to be 413bp according to gene bank accession number AY295085. Sample containing other plasmid DNA was the negative control.

All PCR products were analyzed using a template directed dye-terminator incorporation assay (TDI) with fluorescence polarization (FP). In order to degrade excess dNTP and common conservative primers in the PCR product, 1µL of PCR product, 16.67 nkat of shrimp alkaline phosphatase, 1U of *E. coli* exonuclease I in 1 µL of shrimp alkaline phosphatase buffer (0.5 mol/L Tris-HCl, pH8.5, 50 mmol/L MgCl₂), and 8 µL of distilled water were mixed and incubated at 37°C for 60 min before enzymes were heat-inactivated at 80°C for 15 min. Thirteen microliters of TDI-FP mixture containing 10× reaction

Table 1 *H pylori* infection in the pharynx of patients with chronic pharyngitis

| Group | Case | TDI-FP(%) | Giemsa(%) |
|---------------------------------|------|------------------------|-----------------------|
| Chronic pharyngitis | 50 | 19 (38.0) ^a | 4 (8.0) |
| With stomach ailment history | 16 | 11 (68.8) ^b | 3 (18.8) ^b |
| Without stomach ailment history | 34 | 8 (23.5) | 1 (2.9) |
| Normal (Control) | 20 | 0 | 0 |

^a $P \leq 0.05$ vs Control; ^b $P \leq 0.01$ vs group without stomach ailment history.

buffer, TAMRA-ddTTP, RP110-ddGTP, *H pylori* probes and 7 μ L of the enzymatically treated PCR product were mixed and denatured at 95 °C for 2 min, followed by 25 cycles of 95 °C for 15 s and 50 °C for 30 s. At the end of the cycles, the mixture was held at room temperature. *H pylori* genes were acquired by reading FP (mp) at wavelengths of 535 nm and 595 nm, and analyzed by Fluorescence Polarization-Capable Instrument-Victor.

Statistical analysis

All the data were analyzed by the χ^2 test.

RESULTS

H pylori infections in the pharynx of the people in the control group and the patients suffering from chronic pharyngitis were examined with TDI-FP and modified Giemsa stain. In the control group, analysis with TDI-FP showed that none of the 20 cases was infected with *H pylori* in the pharynx. The examination using modified Giemsa stain revealed that no cases were infected with *H pylori* in the pharynx.

Regarding the 50 cases of the patients who were suffering from chronic pharyngitis, 19 cases (38%) were determined using TDI-FP to be infected with *H pylori* in the pharynx. When the modified Giemsa stain procedure was used, 4 cases (8.0%) of patients were determined to be infected with *H pylori* in the pharynx. In the 16 pharyngitis cases with stomach ailment history, 11 cases (68.8%) were determined using TDI-FP to be infected with *H pylori* in the pharynx, which was remarkably higher ($P \leq 0.01$) than that in the cases without stomach ailment history (8 cases, 23.5%) as determined by the Statistical χ^2 test. From using the modified Giemsa stain method, 3 cases (18.8%) of patients with a stomach ailment history were determined to be infected with *H pylori* in the pharynx, which was remarkably higher ($P \leq 0.01$) than that in patients without a stomach ailment history (1 case, 2.9%), as determined by the statistical χ^2 test (Table 1). This showed the following: (1) that *H pylori* is not detected in the pharynx of healthy people; (2) that chronic pharyngitis is often related to *H pylori* infection; and (3) that a stomach ailment history is associated with a higher rate of *H pylori* infection of the pharynx.

DISCUSSION

We used two methods to detect *H pylori* infection,

template-directed dye-terminator incorporation with fluorescence polarization (TDI-FP), and modified Giemsa stain. TDI-FP is a single base extension technique in which an oligonucleotide probe anneals to a polymerase chain reaction (PCR)-amplified product adjacent to a single nucleotide polymorphism (SNP) of interest^[3,10]. In the presence of DNA polymerase and fluorescently labeled dideoxynucleoside triphosphates (ddNTPs), the probe is extended by a single base dictated by the polymorphic site in the target sequence. Fluorescence polarization, the property that fluorescent molecules emit polarized fluorescent light when excited by plane polarized light, is used to identify incorporated ddNTP, and to assign a genotype^[10]. With this design, the PCR primers will not interfere with the primer extension step of the assay. This method is simple, rapid, sensitive, specific and could also be used for detecting pathogen DNA in the clinic^[11,12]. The modified Giemsa stain is very straightforward, inexpensive, and takes about five minutes to perform, excluding the time in solution, and rarely requires repeat stains^[13].

In the current study, we examined for *H pylori* infection in the pharynx with TDI-FP and the modified Giemsa stain methods. These two methods are rapid, sensitive, specific, easily reproducible and easy to perform technically. The secretion in the pharynx in our study was washed out by water from the epithelial tissue in the checked pharynx before examination so that the results were not affected by gastric juice and saliva.

Sore throat is one of the most common reasons for visits to family physicians. While most patients with sore throat have an infectious cause (such as pharyngitis), fewer than 20 percent have a clear indication for antibiotic therapy (i.e., group A beta-hemolytic streptococcal infection). Useful, well-validated clinical decision rules are available to help family physicians care for patients who present with pharyngitis^[14]. Because of recent improvements in rapid streptococcal antigen tests, throat culture can be reserved for patients whose symptoms do not improve over time or who do not respond to antibiotics. Pharyngitis and the common cold are among the most frequent diseases. Two thirds of the patients consulting for respiratory infection received antibiotic treatment, and antibiotics confer relative benefits in the treatment of sore throat^[16]. Pharyngitis may be caused by a wide variety of microbial agents. *Burkholderia cepacia* is a Gram-negative bacillus that is widely distributed in nature; pharyngitis due to *Burkholderia cepacia* was reported for person-to-person transmission^[17]. The main aetiological agents reportedly causing acute pharyngitis were adenovirus, respiratory syncytial virus (RSV), *Mycoplasma pneumoniae*, *Streptococcus pyogenes* and *Chlamydia pneumoniae*. *M. pneumoniae* was the agent found most frequently as a single pathogen. A history of recurrent pharyngitis, having older siblings and a negative outcome were significantly more common among patients with acute *M. pneumoniae* infection than among those with infections due to other pathogens, or healthy controls. That study demonstrates that: (1) adenovirus and RSV have a prominent role in acute pharyngitis; (2) *S. pyogenes* is found frequently, but it is not possible to distinguish simple carriers from patients with a true infection; and (3) *M. pneumoniae* appears to be

able to cause acute pharyngitis^[18]. In our daily work, we use antibiotics to treat pharyngitis and sore throat and find antibiotics are effective, although we are not clear about the mechanism of the treatment.

H pylori is one of the world's most widespread microorganisms. The abrupt increase of *H pylori* during high school may result from a marked increase of interpersonal social activities^[19]. Its acquisition in humans remains poorly understood, however, epidemiological studies have identified drinking water as a reservoir for the bacterium^[20]. *H pylori* has been associated with the development of gastritis, peptic ulcers and gastric cancer. Although *H pylori* infects up to more than half of the world's population, to date the precise modes of transmission have not been fully understood^[13]. *H pylori* has been investigated in several other organ systems and localizations besides the gastrointestinal cavity, such as the oral cavity. For example, in one study, it was found that the majority of patients with oral diseases have possible *H pylori* colonization in dental plaque; while about two-thirds have *H pylori* associated chronic active gastritis. The oral cavity may be the first place for colonization by *H pylori*, and then the infection involves the gastric mucosa^[21]. Oral hygiene (the frequency of dental visits and teeth cleaning) did not have a significant influence on the presence of *H pylori* in dental plaque. Other investigators supported the hypothesis that *H pylori* infection begins in the oral cavity by concluding that dental plaque is the reservoir of *H pylori* with no relationship to gastric infection^[22]. In our study, the pharynx may be a place for *H pylori* colonization, but the number of *H pylori* was so few that the modified Giemsa stain was not very useful. Therefore, we made an amplification of *H pylori* DNA by using a conservative primer pair, then detected the *H pylori* colonization using TDI-FP and acquired a good result.

Other investigators have studied the localization of *H pylori* as well. Akbayir *et al*^[23] demonstrated that *H pylori* was not present in laryngeal squamous cell carcinoma tissue or in benign lesions. They could not find any evidence indicating that *H pylori* played a role at the tissue level in the pathogenesis of laryngeal carcinoma. Their results may indicate that *H pylori* does not colonize in either adenoid or tonsils and that these tissues do not constitute a reservoir for *H pylori* infection. On the contrary, Cirak *et al*^[7] detected *H pylori* and its *CagA* gene in tonsil and adenoid tissues by PCR. These authors found: 7 of 23 (30%) patients to be positive for *H pylori* DNA, 5 (71%) of whom also possessed the *CagA* gene. In another study, specimens from 208 dyspeptic patients were collected from saliva, supra- and subgingival dental plaque, tongue scrapings, and oropharyngeal swabs. *H pylori* was detected from multiple sites (dental plaque, gastric juice, gastric biopsy, duodenal aspirate, and the oropharynx^[26]). As in our study, the authors used more than one method for detecting *H pylori*. When PCR was used, 15 of 208 patients (7%) tested positively for *H pylori* by PCR in dental plaque. In contrast, only 2 samples were positive by culture. This demonstrates the importance of using more than one method for detecting *H pylori* infection. We also found this to be true in our own study, where we used two methods, TDI-FP and modified Giemsa stain,

to detect *H pylori* infection. As shown in Table 1, TDI-FP consistently detected *H pylori* infections that were missed by the modified Giemsa stain method. These authors also found that four of the dental plaque strains had restriction patterns similar to those of the stomach and duodenal sites, providing evidence that these sites were infected with the same strain of *H pylori*. The detection in dental plaque could indicate that the oral cavity may act as a reservoir or sanctuary for the organism. Likewise, this finding also relates to our data. We found that *H pylori* infection in the pharynx was significantly more likely in patients with a history of stomach ailments than in patients without a history of stomach ailments. That is, just as there is a relationship between the occurrence of *H pylori* in dental plaque and its occurrence in the stomach and duodenum, there may be a relationship between infection of the pharynx with *H pylori* and a history of stomach ailments. However, whether *H pylori* is a resident or transient oral microorganism is still unclear, although it is more likely to be transient in nature^[27].

In our study, the goal was to determine if there was a relationship between *H pylori* infection in the pharynx and chronic pharyngitis, and, in addition, to determine if such infections correlated with a history of stomach ailments. We determined both of these relationships to be true. In addition, we utilized two methods for detecting *H pylori*, TDI-FP and modified Giemsa stain, and found the TDI-FP method to be the most sensitive. These findings can be applied to the clinical setting: doctors will be able to use the appropriate method to test if a patient with chronic pharyngitis is infected in the pharynx with *H pylori*, aiding in prescribing the appropriate antibiotic. These findings also should cause doctors to examine a patient with chronic pharyngitis for ailments in the digestive system. But on the other hand, the TDI-FP test can yield false positive results.

To determine whether *H pylori* infection is related to other upper respiratory tract infections, such as sinusitis, or with otitis media, further investigations will need to be performed.

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Heat-shocked tumor cell lysate-pulsed dendritic cells induce effective anti-tumor immune response *in vivo*

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(24 mm³ vs 8 mm³, $P=0.480$). The median survival time of mice immunized with HSCT-26 DCs was longer than that of those immunized with CT-26 DCs (57 d vs 43 d, $P=0.0384$).

CONCLUSION: Heat-shocked tumor cell lysate-pulsed DCs can evoke anti-tumor immune response *in vivo* effectively and serve as a novel DC-based tumor vaccine.

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Key words: Heat shock; Tumor; Dendritic cell; Immune

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Abstract

AIM: To study whether heat-shocked tumor cells could enhance the effect of tumor cell lysate-pulsed dendritic cells (DCs) in evoking anti-tumor immune response *in vivo*.

METHODS: Mouse undifferentiated colon cancer cells (CT-26) were heated at 42°C for 1 h and then frozen-thawed. The bone marrow-derived DCs pulsed with heat-shocked CT-26 cell lysate (HSCT-26 DCs) were recruited to immunize syngeneic naïve BALB/c mice. The cytotoxic activity of tumor specific cytotoxic T lymphocytes (CTLs) in mouse spleen was evaluated by IFN-enzyme-linked immunospot (ELISpot) and LDH release assay. The immunoprophylactic effects induced by HSCT-26 DCs in mouse colon cancer model were compared to those induced by single CT-26 cell lysate-pulsed DCs (CT-26 DCs) on tumor volume, peritoneal metastasis and survival time of the mice.

RESULTS: Heat-treated CT-26 cells showed a higher hsp70 protein expression. Heat-shocked CT-26 cell lysate pulsing elevated the co-stimulatory and MHC-II molecule expression of bone marrow-derived DCs as well as interleukin-12 p70 secretion. The IFN- γ secreting CTLs induced by HSCT-26 DCs were significantly more than those induced by CT-26 DCs ($P=0.002$). The former CTLs' specific cytotoxic activity was higher than the latter CTLs' at a serial E/T ratio of 10:1, 20:1, and 40:1. Mouse colon cancer model showed that the tumor volume of HSCT-26 DC vaccination group was smaller than that of CT-26 DC vaccination group on tumor volume though there was no statistical difference between them

INTRODUCTION

Dendritic cells (DCs) are the most potent antigen-presenting cells (APCs) mediating effective immune effects *in vitro* and *in vivo*^[1,2]. DCs-based tumor vaccines have been recruited to prevent postoperative recurrence and metastasis of malignant tumors^[3,4]. During the development of this approach, distinct methods have been attempted to enhance the effect of DC tumor vaccine in evoking tumor rejection response.

Heat shock protein (HSP), the molecule chaperon in cells, binds to the antigenic peptides and guides them to accurate folding, transporting, and conjugating to major histocompatibility complex (MHC) molecules^[5]. Referring to the current knowledge of HSPs, it acts as chaperone peptides including antigenic peptides, interacts with antigen presenting cells through a receptor, stimulates antigen presenting cells to secrete inflammatory cytokines and mediates maturation of DCs. It was reported that heating could enhance the immunogenicity of tumor cells, which is ascribed to HSPs^[6]. Furthermore, vaccination with the lysate of heated tumor cells can result in effective tumor rejection *in vivo*^[7]. In the present study, we used heat-shocked tumor cells to elicit HSP expression. We assumed that pulsing with these HSP-rich tumor cell lysate might enhance the effect of DCs to stimulate stronger anti-tumor response than pulsing with tumor cell lysates. Because hyperthermal treatment is a standardized manipulation in

clinical practice of oncologic surgery, such a DC tumor vaccine is convenient to be prepared. We examined the specific CTL response induced by heat-shocked tumor cell lysate-pulsed DCs in naïve mice and further evaluated its immunoprophylactic effects in mouse colon cancer model.

MATERIALS AND METHODS

Mice and cell line

Six to eight-week-old female BALB/c mice were purchased from Laboratory Animal Research Center (LARC) of the Fourth Military Medical University (FMMU, Xi'an) and housed under pathogen-free conditions. All experiments involving the use of mice were performed in accordance with the protocols approved by LARC. CT26 is a carcinogen-induced undifferentiated colon adenocarcinoma cell line of BALB/c mice. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% heat-inactivated fetal bovine serum (FBS; Sijiqin Biotech, Hangzhou), 100 IU/mL penicillin and 100 µg/mL streptomycin at 37 °C in an atmosphere containing 50 mL/L CO₂ and passaged every 2 d.

Heating and lysing CT-26 cells

CT-26 cells at 90% confluence were heated in 42 °C water bath for 1 h followed by recovering for 2 h at 37 °C in an atmosphere containing 50 mL/L CO₂. Cells were then digested by 0.02% trypsin and washed twice with PBS. After being enumerated and re-suspended in PBS at 1×10⁶ cells/mL, CT-26 cells were frozen in liquid nitrogen for 10 min and then thawed thrice at 4 °C. The frozen-thawed resultants were centrifuged at 12 000 *g* for 15 min and the supernatant was preserved as tumor cell lysate at -80 °C.

Western blot assay

Equivalent protein samples were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Then, the separated proteins were transferred onto nitrocellulose (NC) membranes. Membranes were blocked with 5% non-fat milk in TBST (20 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, and 0.05% Tween 20) for 2 h and incubated for 1 h at room temperature with hsp70 mAb (H5147, Sigma) diluted in TBST. After washing, membranes were incubated with horseradish peroxidase conjugated goat-anti-rabbit IgG (Boxter) diluted 1:400 in TBST at room temperature for 1 h. Detection was performed using the DAB detection system.

Generation and pulse of bone marrow-derived DCs

Bone marrow-derived DCs were generated as described by Lutz *et al*^[8] with minor modifications. Briefly, 1×10⁶ cells/mL erythrocyte-depleted mouse bone marrow cells from flushed marrow cavities were cultured in complete medium (CM) with 20 ng/mL recombinant mouse GM-CSF (PeproTech, Rocky Hill, NJ, USA) in 10-cm tissue culture dishes at 37 °C in an atmosphere containing 50 mL/L CO₂. On d 3, 5, and 7, respectively, half media were removed and centrifuged for 5 min at 1 500 r/min, the collected cells were resuspended in the same volume of fresh CM and replenished to original plates. The frozen-

thawed tumor lysate was added to the DC culture systems on d 7 at a ratio of five DC equivalents to one tumor cell (i.e., 5:1) and incubated at 37 °C in an atmosphere containing 50 mL/L CO₂. After 48 h of incubation, non-adherent cells including DCs were harvested by gentle pipetting. DCs were enumerated by FACS (FACScan, Becton Dickinson) analysis through staining with PE anti-mouse CD11c Ab (N418, hamster IgG, Biolegend). The co-stimulatory and MHC-II molecules were analyzed by staining with FITC anti-mouse I-A/I-E (m5/114.15.2, ratIgG2b, Biolegend) and FITC anti-mouse CD86 (B7-2, PO3, ratIgG2b, Biolegend). The corresponding labeled isotypes served as the controls. For further vaccination, DCs were washed twice, enumerated and resuspended in PBS at 5×10⁶/mL.

ELISA

Following the protocol of mIL-12 p70 ELISA Ready-SET-Go kit (Ebioscience, San Diego, CA, USA), plates (NUNC Maxisorp) were pre-coated with capture antibody (clone C18.2) overnight at 4 °C and blocked at room temperature for 1 h. After being washed, 100 µL/well of mIL-12 p70 standard (8 pg-1 024 pg/mL at twofold serial dilutions) or 100 µL/well of samples was added to the appropriate wells and incubated at room temperature for 2 h. Wells were aspirated and washed, then 100 µL/well detection antibody (clone C17.8) was added and incubated at room temperature for 1 h. After being washed, 100 µL avidin-HRP was added and incubated at room temperature for 30 min. Plates were washed thoroughly and 100 µL/well of substrate solution was added. After being incubated at room temperature for 15 min, 50 µL of stop solution was added to each well. The plates were read. Data represented the value of 450 nm subtracted the value of 570 nm. All assays were performed in triplicate.

Immunization assay and colonic cancer inoculation

BALB/c mice were immunized with DC vaccine through tail-vein on d 0 and 7 respectively at the same dose of 5×10⁵ DCs (100 µL). Each treatment group contained not less than 15 mice. Seven days after the second immunization, three mice of each group were killed and the spleens were taken to perform the ELISpot and cytotoxic assays. The remaining mice were anesthetized with 0.75 mg of sodium pentobarbital. Colonic cancer inoculation was performed as follows. In brief, a vertical midline incision was made and the cecum was exposed. Upon visualization, 1×10⁵ CT-26 cells (50 µL) were injected into subserosa using a 30-gauge needle (Becton Dickinson) and 1 mL syringe. The midline incision was closed with a running suture. Fourteen days later, not less than five mice of each group were killed, the weight of the colon tumors was measured and the peritoneal metastases were checked. The remaining mice were fed to observe the tumor-bearing survival time. The end point of observation was selected when all the mice of any of the two teams were dead.

ELISpot analysis

The murine interferon-gamma ELISpot kit (Diaclone, France) was used to determine tumor-specific IFN-γ

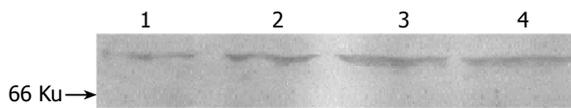


Figure 1 Western blot detection of HSP70 expression of CT-26 cells heating at 42 °C. 1: No heating. 2: heat-shocked for 30 min. 3: heat-shocked for 1 h. 4: heat-shocked for 2 h.

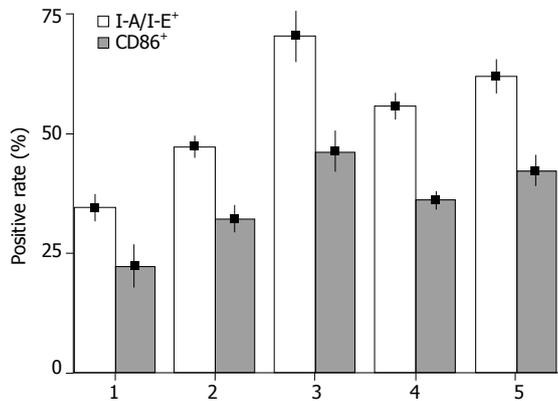


Figure 2 The surface molecule expressions of mouse bone marrow derived DCs were measured by staining with PE anti-mouse CD11c (N418, hamster IgG), FITC anti-mouse I-A/I-E (m5/114.15.2, ratIgG2b), FITC anti-mouse CD86 (B7.1, PO3, ratIgG2b) and analyzed by FACS. 1: DC culture system at d 7. 2: DC culture system at d 9. 3: DC culture system stimulating with LPS(1 µg/mL). 4: DC culture system pulsed with CT-26 lysates. 5: DC culture system pulsed with HSCT-26 lysates. The data are representative for 3 independent experiments.

secreting T cells^[9]. The 96-well filtration plates (Nunc) were coated with 100 µL capture antibody (clone DB1). After an overnight incubation at 4 °C, the wells were washed and blocked with 2% dry skimmed milk in PBS. Splenocytes (1×10^6 /well) isolated from the mice were added to the wells and incubated at 37 °C in an atmosphere containing 50 mL/L CO₂ for 20 h with target cells (5×10^4 /well). Plates were washed and then incubated with 100 µL biotinylated detection antibody (polyclonal) at 37 °C for 90 min. After the removal of unbound antibodies, 100 µL avidin-alkaline phosphatase was added and plates were incubated for 1 h at 37 °C. After washing, spots were developed by adding 100 µL of ready-to-use BCIP/NBT buffer and incubated at room temperature for spot formation. The spots were scanned and counted.

Lactate dehydrogenase release assay

Specific cytolytic activities of murine spleen cells were determined by LDH assay. The CT-26 cells were used as target cells. Effector and target cells were mixed at the E/T ratio of 20:1 at 0.2 mL/well in 96-well round-bottomed plates (Nunc). After incubation for 4 h, cells were centrifuged at 250 r/min for 5 min and the cell-free supernatant was collected for LDH assay using CytoTox96 (Promega, Madison, WI, USA). The percentage of specific LDH release was calculated by the following formula: % cytotoxicity = [(experimental LDH release)-(spontaneous LDH release by effector and target cells)/(maximum LDH release) × (spontaneous LDH release)] × 100. For the controls, the target cells were incubated either in culture

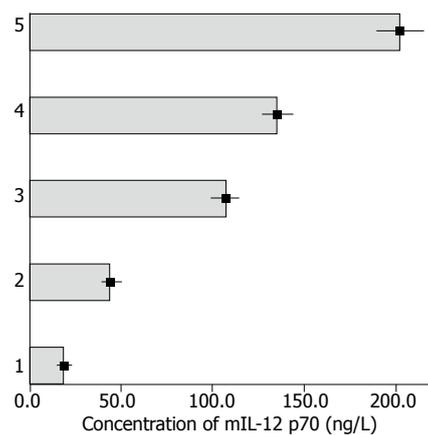


Figure 3 The concentration of mIL-12 p70 in supernatant of different DC culture system were measured by ELISA assay. 1: DC culture system at d 7. 2: DC culture system at d 9. 3: DC culture system pulsed with CT-26 lysates. 4: DC culture system pulsed with HSCT-26 lysates. 5: DC culture system stimulating with LPS (1 µg/mL). The data were representative for three independent experiments.

medium alone to determine spontaneous release or in a mixture of 2% Triton X-100 to define the maximum LDH release. The spontaneous release was always <10% of the maximum release. All assays were performed in triplicate.

Statistical analysis

Each group included at least five mice. All experiments were carried out thrice. Data were expressed as mean ± SD. Tumor volume and immune cell yield data were analyzed by two-way analysis of variance (ANOVA). All analyses were conducted with SPSS8.0 software. $P < 0.05$ was considered statistically significant.

RESULTS

Phenotype and cytokine production in heat-shocked CT-26 cell lysate-pulsed DCs

To evaluate the hsp70 protein expression in CT-26 cells, we performed Western blot analysis using an anti-hsp70 mAb. hsp70 protein in CT-26 cells increased after being heated for 30 min and peaked at 1 h (Figure 1). Therefore, heating at 42 °C for 1 h was selected as a standard treatment for heat shocking in the following experiment. To determine the quantity of DCs in culture system, we analyzed the surface molecules of harvested cells by flow cytometry. About 65% of the harvest cells in culture system on d 7 were CD11c positive and increased to 75% or more on d 9. DCs pulsed with HSCT-26 lysate manifested higher CD86 and I-A/I-E expression than pulsed with CT-26 lysate (Figure 2). We also measured the mIL-12 p70 concentration in the supernatant of different DC culture systems by ELISA. HSCT-26 lysate-pulsed DCs showed a higher level of mIL-12 p70 than either CT-26 lysate-pulsed or unpulsed DCs on d 9 (Figure 3).

Immunization with HSCT-26 lysate-pulsed DCs elicited tumor specific CTLs in naïve BALB/c mice

To investigate the ability of HSCT-26 lysate-pulsed DCs to induce CT-26 specific CTLs in naïve syngeneic BALB/c mice, we examined the CT-26 specific IFN-γ-producing T

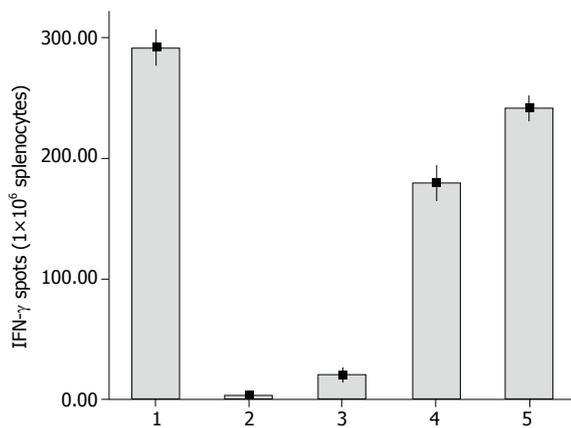


Figure 4 Tumor-specific IFN- γ secreting Splenic T cells induced by different cell lysates pulsed DC. 1: positive control (no target, 1mg/L PHA stimulated). 2: negative control (no target). 3: single DC immunized. 4: CT-26 lysates pulsed DC immunized. 5: HSCT-26 lysates pulsed DC immunized. Results are representative of three experiments.

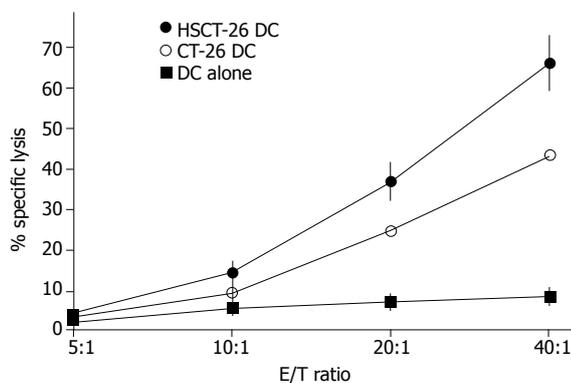


Figure 5 Bone marrow-derived DC pulsed with HSCT-26 lysate can generate tumor-specific CTLs *in vivo*. Splenic T cells obtained from immunized mice were evaluated for cytolytic activity against Mitomycin C treated CT-26 cells at the effector: target (E:T) ratios indicated. Values are the mean \pm SE of triplicate wells.

cells in splenocytes derived from immunized and control mice by ELISpot assay. As shown in Figure 4, after being restimulated with mitomycin C-treated CT-26 cells at an E:T ratio of 20:1, the CT-26 specific IFN- γ secreting T cells in HSCT-26 DCs-immunized mice were significantly more than those in CT-26 DCs-immunized mice ($P=0.002$). Then we evaluated the CT-26 specific cytolytic activity of splenocytes stimulated with different DC tumor vaccines. As shown in Figure 5, splenocytes from mice that received HSCT-26 DC vaccination displayed significantly stronger cytolytic activity against CT-26 cells at ratios 10:1, 20:1, and 40:1 of effector cells to target cells.

Immunization of HSCT-26 lysate-pulsed DCs protected mice with colonic cancer inoculation

We examined whether heat-shocked CT-26 cells lysate-pulsed DCs could display enhanced immunoprophylactic potential in mouse colon cancer model. After being vaccinated twice at an interval of 7 d, BALB/c mice were inoculated with CT-26 cells into cecum through open surgery. Fourteen days after the inoculation, autopsies were performed to check the growth and metastasis of colon

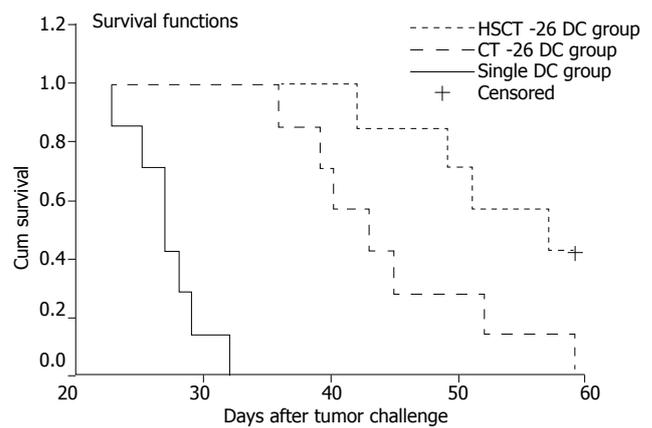


Figure 6 Survival curves of mice immunized with different dendritic cells tumor vaccine.

tumor. The mice that received CT-26 DC vaccination or single DC vaccination had colon tumor formation, whereas one of the six mice that received HSCT-26 DC vaccination was tumor free. The tumor volumes in single DC vaccination group, CT-26 DC vaccination group and HSCT-26 DC vaccination group were 107 ± 69 , 24 ± 8 , and 8 ± 7 mm³, respectively. ANOVA analysis showed significant differences in tumor volume among the three groups ($P=0.000$), but there was no difference between HSCT-26 DC vaccination group and CT-26 DC vaccination group ($P=0.480$). Neither HSCT-26 DC vaccination group nor CT-26 DC vaccination group had peritoneal metastasis. In contrast, 50% mice in single DC vaccination group (3/6) had tumor planting to adjacent peritoneum and intestine surface accompanied with a small quantity of bloody ascites. No hepatic metastasis was found in the mice of the three groups macroscopically during autopsy.

To investigate the tumor-bearing survival time, we observed immunized mice for at least 60 d. The median survival time of mice in the single DC vaccination group, CT-26 DC vaccination group and HSCT-26 DC vaccination group was 27.0, 43.0, and 57.0 d, respectively. Log-rank test showed that the median survival time was significantly different in the three groups of mice ($P=0.0001$). Most importantly, the mice vaccinated with HSCT-26 DCs had a much longer survival time than those vaccinated with CT-26 DCs ($P=0.0384$). The survival curves are shown in Figure 6.

DISCUSSION

Radical surgery is the standard treatment for most patients with advanced cancer. After resection of primary tumor and local drainage lymph nodes, the tumor burden of patients is released and the immune system may have a good chance for recovery. It is the critical time to commence adjuvant immunotherapy for preventing postoperative recurrence and metastasis. The prophylactic effects of this therapy last for a long time and can be augmented by revaccination.

The anti-tumor effect of tumor cell lysate-pulsed DCs was first reported in 1998^[10] and has been utilized to treat

malignant diseases including renal cancer, lymphoma, and colorectal cancer, *etc.*^[11-13]. However, a misgiving of rising autoimmunity still exists as shown in animal models^[14]. Current pilot clinical studies have not found clinical signs of autoimmune disease except for vitiligo and occurrence of auto antibodies. Reinhard *et al.*^[15] reported that vaccination with tumor lysate-pulsed DCs does not show a higher incidence of autoimmunity than vaccination with peptide-pulsed DCs. Tumor lysate is superior to other ways of DC pulsing and can elicit immunity for the entire array of TAAs of source tumors, circumventing the need of prior identification of TAAs from individual cancers. Enhancing the anti-tumor effect of whole tumor cell lysate-pulsed DCs promotes the use of this kind of tumor vaccine.

In our present study, tumor cells heated at 42 °C increased the expression of HSPs such as HSP70. Pulsed with such lysates of heat-shocked tumor cells, DCs showed higher expression of MHC-II and co-stimulatory molecules on their surface and secreted more mIL-12 P70. As we know, DC priming T lymphocytes require signals I and II. Signal I presents the antigenic epitopes introduced by MHC molecules. Signal II as the co-stimulatory molecules on DC surface, can improve epitopes of DCs by interacting with their respective ligands on T lymphocytes. Upregulated expression of MHC-II and co-stimulatory molecules on DC surface facilitates activation of T lymphocytes effectively. IL-12 is a cytokine leading to cell-mediated immune response (Th1 response) and is preferable for successful tumor immunotherapy^[16]. Heat-induced HSPs may also serve as endogenous danger signals to drive DC maturation. Mature DCs obtain high viability and present antigenic epitopes to T lymphocytes through migrating to local drainage lymph nodes. Our result is in agreement with related reports^[17]. DC activating process may be involved in NF- κ B pathway^[18].

It is believed that HSP-chaperoned TAAs represent the specific fingerprint of tumor cells. A gp96 receptor, CD91 on APC surface has been recently identified^[19,20]. HSP-chaperoned antigen might target to APCs with the assistance of HSP receptors on these cells. HSP-peptide complexes can be internalized by APCs through receptor-mediated endocytosis^[21]. HSP-mediated epitopes could go through endogenous antigen-processing pathways in the context of MHC- I molecules^[22]. DCs have an impressive ability to “cross-present” MHC class I-restricted peptide epitopes derived from exogenous proteins to CD8 T lymphocytes. In our study, compared to tumor cell lysate-pulsed DCs, immunization with heat-shocked tumor cell lysate-pulsed DCs might induce a large amount of specific CTLs in naïve mice *in vivo*. Furthermore, the induced CTLs exhibited a higher tumor specific cytotoxic activity. Immunized and challenged mouse model proved that vaccination of HSCT-26 DCs could prolong the survival time of tumor-bearing animals. All the results suggest that heating tumor cells can enhance the effect of whole tumor cell lysate-pulsed DCs to elicit specific anti-tumor response. We suppose that after taking up the HSP-chaperoned antigens, epitopes coming from HSCT-26 might be complexed with MHC class I molecules in endoplasmic reticulum and transported to the cell surface

to priming CD8 T lymphocytes. Increased HSPs in tumor cells may serve as epitope chaperons and natural adjuvants in the process of DC antigen presentation, enhancing anti-tumor effects of DC vaccine.

In conclusion, heat-shocked tumor cell lysate-pulsed DCs can elicit effective anti-tumor effects *in vivo*, suppress the growth of colon cancer and eliminate occurrence of peritoneal metastasis. Using this tumor vaccine postoperatively may decrease the local recurrence and peritoneal metastasis of colon cancer and prolong the tumor-free survival time of the patients. The prophylactic effects of this DC vaccine on preventing liver metastasis of colon cancer should be further evaluated.

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Intestinal permeability in rats with CCl₄-induced portal hypertension

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Abstract

AIM: To investigate the intestinal barrier changes in rats with CCl₄-induced portal hypertension.

METHODS: The permeability of intestinal barrier detected by Lanthanum as a tracer was evaluated in rats. Bacterial translocation and plasma endotoxin were also determined.

RESULTS: The incidence of bacterial translocation was 85% in rats with CCl₄-induced portal hypertension, which was significantly higher than that in control rats (20%, $P < 0.01$). Plasma endotoxin level was significantly higher in experimental group than in control group. Permeability of the epithelial mucosa and pathological alteration were increased in the ileum and the microvilli became shorter and thinner in rats with portal hypertension.

CONCLUSION: Bacterial translocation occurs in rats with CCl₄-induced portal hypertension and increased permeability between epithelial cells contributes to the translocation.

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Key words: Portal hypertension; Bacterial translocation; Intestinal barrier

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INTRODUCTION

The incidence of bacterial infection in patients with portal

hypertension is higher than that in the general population^[1]. Most of these infections are caused by bacteria normally present in the intestine. Portal hypertension may play an important role in the pathogenesis of infections in cirrhotic patients^[2]. On the other hand, endotoxemia present in patients with portal hypertension is correlated with the severity of the disease. Recently, bacterial translocation from the gut to extraintestinal organs and systemic circulation has been proved in patients with other diseases^[3] and is considered as the cause of endotoxemia in patients due to the disruption of the gut barrier function. However, the exact route of bacterial translocation and endotoxemia in patients with portal hypertension is not clear. In the present study, bacterial translocation, permeability of the enterocyte membrane and intestinal morphological alterations in rats with CCl₄-induced portal hypertension were evaluated by transmission electroscopy.

MATERIALS AND METHODS

Adult male Sprague-Dawley rats ($n = 40$) weighing 250-300 g were used. The animals were allowed to acclimate to laboratory condition for 4-6 d with free access to water and rat chow before the experiment.

Twenty rats entered the experimental group and received intragastric CCl₄ via a Hamilton syringe with an attached stainless steel animal feeding tube after light ether anesthesia. The first dose of CCl₄ was 20 μ L and subsequent doses were adjusted based on the body weight 48 h after the last dose. After the appearance of ascites, the dose was reduced to 40 μ L and increased according to the schedule if ascites resolved. The other 20 rats were gavaged with water and served as control group.

Studies on intestinal permeability and bacterial translocation from the gut were carried out on d 14. The abdominal cavity was opened by a midline incision and blood samples were immediately inoculated into 3 mL blood culture medium and 0.5 mL plasma was used to detect endotoxin. Limulus-amoebocyte-lysate (LAL) test was used to determine plasma endotoxin levels as previously described^[4]. *Escherichia coli* 055 was used as endotoxin standard. The results were expressed as endotoxin units per milliliter (EU/mL). The mesenteric lymph nodes (MLNs) near the distal ileum, liver, and spleen were harvested and weighed, tissues were then homogenized in a test tube containing 3 mL brain heart infusion broth, 0.2 mL supernatant was taken for bacterial culture. All media for aerobic culture were incubated at 37 °C for

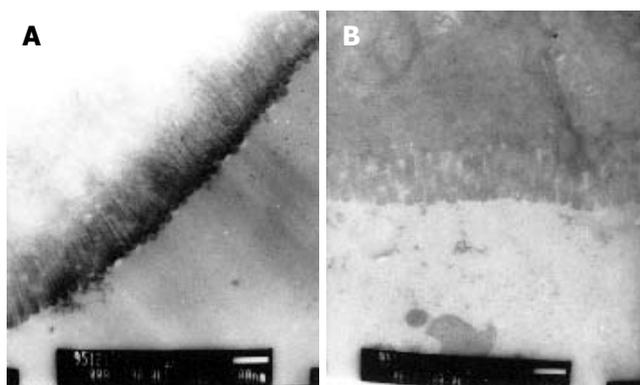


Figure 1 Normal microvilli of enterocytes in control rats (A) and shortened and thinned microvilli of enterocytes in rats with portal hypertension (B).

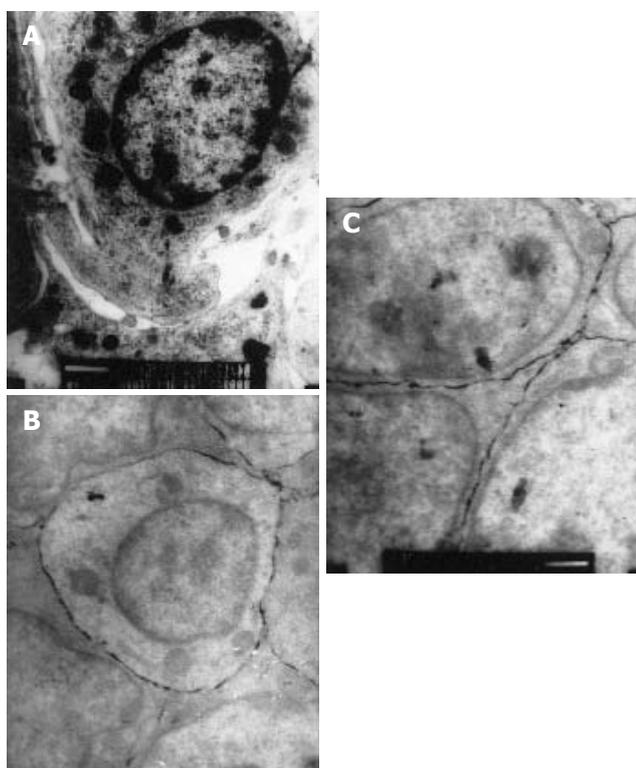


Figure 2 Lanthanum tracer in the ileum of control rats (A) and rats with CCl₄-induced portal hypertension (B and C).

3-5 d and isolated bacteria were identified by standard procedures. At the end of the experiment, a segment of the distal ileum was removed and placed into 2.5% glutaraldehyde fixative with 4% lanthanum hydroxide for 1 h, then washed thrice to assay the permeability of the intestinal barrier. Sections were made from each sample and the presence of the tracer was observed under transmission electron microscope.

Positive MLN culture was considered indicative of bacterial translocation from the gut. Positive blood, spleen or liver cultures were considered indicative of passage bacteria to the systemic circulation.

Statistical analysis

Translocation incidence was analyzed by the Fisher's exact test. Weights and other data were expressed as mean \pm SD

and compared with Student's *t* test.

RESULTS

The mean spleen weight in rats with CCl₄-induced portal hypertension (5.32 ± 0.38 mg/g body wt) was significantly greater than that in control rats (2.31 ± 0.28 mg/g body wt). The weight of MLN was also greater in rats with CCl₄-induced portal hypertension (1.85 ± 0.42 mg/g body wt) than that in control rats (0.86 ± 0.25 mg/g body wt). Ascites occurred in all rats.

Bacterial translocation to MLN occurred in 17 of 20 (85%) CCl₄-PH rats and in 4 of 20 (20%) control rats. Bacteria were found in ascites in the experimental group (7/20, 35%) while no bacteria were found in the control group. All bacteria isolated from MLNs and ascites were Gram-negative. No bacteria were isolated from blood, spleen or liver.

Plasma levels of endotoxin (0.083 ± 0.012 EU/mL) and ascites (0.062 ± 0.012 EU/mL) were significantly higher in the experimental group than in the control group (0.046 ± 0.009 EU/mL, $P < 0.01$).

The distal ileum of rats with CCl₄-induced portal hypertension did not differ grossly from that of the control rats. Clear and intact microvilli were seen in ileal enterocytes in the control rats. Shortened and thinned microvilli appeared in rats with CCl₄-induced portal hypertension (Figure 1).

The tracer did not penetrate into the cells or intercellular juncture in the control rats. The tracer appeared between enterocytes and cellular junctions in rats with CCl₄-induced portal hypertension. The penetrated tracer aggregated along the cellular junctions (tight or gap) or within the mucosal propria lamina without entering enterocytes or winding lines (Figure 2).

DISCUSSION

The mechanism underlying bacterial penetration into the intestinal barrier and entry into the systemic circulation in cirrhotic patients remains unclear^[5-7]. Recent studies in animal model demonstrated that bacteria from the gastrointestinal tract can cross over the intestinal barrier to infect extraintestinal sites and/or the systemic circulation, a process known as bacterial translocation^[8-10]. Various pathological changes such as damage of the intestinal barrier can increase the incidence of bacterial translocation^[11]. Cirrhotic patients usually develop portal hypertension that causes intestinal congestion, thus inducing intestinal mucosal abnormalities^[12], suggesting that portal hypertension may play a prominent role in the pathogenesis of spontaneous bacteremia and peritonitis in cirrhotic patients. Our results indicated that bacterial translocation occurred in 85% portal hypertensive rats, which was significantly higher (20%) than that in the control rats. Surgical manipulation and stress may be responsible for the translocation of bacteria^[13-15].

The reason why permeability of the intestinal mucosa is increased remains unclear. In the present study, the tracer (Lanthanum) with a mean diameter of 4 nm is normally located outside the cell membrane, but may

enter the cells through cellular pores, while their diameter exceeds 2 nm. Our results showed that the permeability of intestinal barrier was greatly increased in rats with portal hypertension. The tracer appeared between enterocytes and lamina propria cells, indicating that translocation occurs between enterocytes and lamina propria cells. This finding is consistent with the observation of Cole *et al.*^[17]. The damaged microvilli in rats with CCl₄-induced portal hypertension demonstrated that permeability changes might occur before the development of pathological abnormality^[15-17].

In conclusion, bacterial translocation occurs in rats with CCl₄-induced portal hypertension and the increased permeability between epithelial cells contributes to the translocation.

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RAPID COMMUNICATION

Serum soluble interleukin-2 receptor levels in patients with chronic hepatitis B virus infection and its relation with anti-HBc

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Abstract

AIM: To investigate the relationship between serum soluble interleukin-2 receptor (sIL-2R) level and anti-HBc in patients with chronic hepatitis B virus (HBV) infection.

METHODS: Sera from 100 patients with chronic HBV infection and 30 healthy controls were included in this study. The patients were divided into group A [HBsAg (+), HBeAg (+) and anti-HBc (+), $n = 50$] and group B [HBsAg (+), HBeAg (+) and anti-HBc (-), $n = 50$]. sIL-2R levels were determined using ELISA. HBV DNA and alanine aminotransferase (ALT) were also detected.

RESULTS: Serum sIL-2R levels were significantly higher in patients with chronic HBV infection than in healthy controls. Moreover, serum sIL-2R levels were significantly higher in patients with HBsAg (+), HBeAg (+) and anti-HBc (+) ($976.56 \pm 213.51 \times 10^3$ U/L) than in patients with HBsAg (+), HBeAg (+) and anti-HBc (-) ($393.41 \pm 189.54 \times 10^3$ U/L, $P < 0.01$). A significant relationship was found between serum sIL-2R and ALT levels ($P < 0.01$) in patients with chronic HBV infection, but there was no correlation between sIL-2R and HBV DNA levels. The anti-HBc status was significantly related to the age of patients ($P < 0.01$).

CONCLUSION: The high sIL-2R level is related to positive anti-HBc in chronic hepatitis B patients. Positive anti-HBc may be related to T-lymphocyte activation and negative anti-HBc may imply immune tolerance in these patients.

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Key words: Chronic hepatitis B; Hepatitis B virus; Anti-

INTRODUCTION

About 350 million persons are chronically infected with hepatitis B virus (HBV) in the world^[1]. Carriers of HBV are at an increased risk of developing cirrhosis, hepatic decompensation and hepatocellular carcinoma (HCC)^[2]. China has the greatest burden of hepatitis B and liver cancer in the world. A third of all chronic HBV carriers live in China. Each year, about half a million Chinese die of liver cancer or liver failure due to hepatitis B. However, HBV has no cytopathic effect on hepatocytes. Some liver damages caused by HBV are attributed to immune clearance of virus-infected cells and associated immune reactions. While antibody response in patients with HBV infection plays a critical role in viral clearance through the formation of complexes with viral particles and their removal from the circulation^[3], specific cellular immune response plays a main role in hepatic necrosis due to HBV infection and in the persistence of viral infection^[4].

IL-2R system plays an important role in the activation and proliferation of lymphocytes^[5]. IL-2R is expressed on the cell membrane of lymphocytes and contains at least three different chains. Serum sIL-2R is predominantly released from activated T lymphocytes and can serve as an index of activation of T lymphocytes^[6]. Serum sIL-2R levels are significantly higher in patients with chronic HBV infection than in healthy controls^[7]. The serum sIL-2R level one year after interferon administration may be a useful marker of interferon's therapeutic effectiveness^[8].

In the present study, we determined the serum levels of sIL-2R in chronic hepatitis B patients with positive or negative anti-HBc to analyze the elevated patterns of sIL-2R in patients with different anti-HBc status.

MATERIALS AND METHODS

Patients

Serum samples were obtained from 100 Chinese patients

Table 1 Levels of sIL-2R, ALT, and HBV DNA in sera of patients with chronic HBV infection (mean \pm SD)

| Group | Number | sIL-2R ($\times 10^3$ U/L) | ALT (IU/L) | HBV DNA (copies/mL, IgC) |
|---------|--------|----------------------------------|----------------------------|-----------------------------|
| A | 50 | 967.56 \pm 213.51 ^b | 79 \pm 21.2 ^d | 7.954 \pm 1.754 |
| B | 50 | 393.41 \pm 189.54 ^a | 24 \pm 12.3 | 7.875 \pm 1.011 |
| Control | 30 | 243.59 \pm 121.34 | 13 \pm 6.5 | 0 |

^b $P < 0.01$ vs group B and control group; ^a $P < 0.05$ vs control group; ^d $P < 0.01$ vs group B and control.

positive for HBsAg and HBeAg. All the patients were followed up from 2001 to 2004. The patients were divided into two groups. Group A consisted of 50 patients positive for HBsAg, HBeAg, and anti-HBc (age range, 3-71 years; mean age, 37 years). Group B consisted of 50 patients positive for HBsAg and HBeAg but negative for anti-HBc (age range, 12-33 years; mean age, 22.5 years). Another 30 healthy persons negative for all HBV markers served as the control group.

Blood sampling

Venous blood samples were taken to detect sIL-2R, alanine aminotransferase (ALT), HBV DNA and HBV markers (HBsAg, HBeAg, and anti-HBc). All serum samples were separated and stored at -20 °C until testing.

Determination of sIL-2R

Serum sIL-2R levels were determined by sandwich ELISA using commercially available sIL-2R assay kits (Department of Immunology, Dr Bethune Medical University, Changchun, China). An anti-sIL2R monoclonal antibody was adsorbed onto the substrate of polystyrene microtiter wells. The sIL-2R present in the samples or in the standard solutions was bound to the antibody-coated wells. The unbound sample components were removed by washing thrice. A peroxidase-linked anti-sIL-2R monoclonal antibody against another epitope on the sIL-2R molecule was then added to complete the sandwich. After being washed, the unbound materials were removed and a substrate solution was added into the wells. A stopping solution was added to stop the reaction and then light absorbance at 492 nm was measured. A standard curve was prepared from four IL-2R standards. The values were expressed as unit (U) per L.

Assay of HBV markers and HBV DNA

HBsAg, HBeAg, and anti-HBc were detected using commercially available EIA or ELISA kits (Reagents Development Center, Shanghai Hospital for Infectious Diseases, Shanghai, China). HBV DNA levels were tested using real-time PCR on ABI 7000 real-time detection system (Applied Biosystems, Foster City, CA, USA).

Measurement of ALT

ALT was tested on a CX4 chemistry analyzer (Beckman Coulter, Fullerton, CA, USA) using commercially available kits.

Table 2 Age difference between anti-HBc (+) and anti-HBc (-) patients with chronic HBV infection

| Group | Number | Median (yr) | Rank sum |
|-------|--------|-----------------|----------|
| A | 50 | 37 ^b | 66.88 |
| B | 50 | 22.5 | 40.12 |

^b $P < 0.0001$ vs group B.

Statistical analysis

The significance of difference between the two groups was determined with Student's *t* test and Wilcoxon's rank-sum test. $P < 0.05$ was considered significant.

RESULTS

Serum sIL-2R levels were significantly higher in patients with chronic HBV infection than in healthy controls. Moreover, serum sIL-2R levels were significantly higher in patients with HBsAg (+), HBeAg (+) and anti-HBc (+) (group A, 976.56 \pm 213.51 $\times 10^3$ U/L) than in patients with HBsAg (+), HBeAg (+) and anti-HBc (-) (group B, 393.41 \pm 189.54 $\times 10^3$ U/L, $P < 0.01$). ALT levels were significantly higher in group A (79 \pm 21.2 $\times 10^3$ U/L) than in group B (24 \pm 12.3 $\times 10^3$ U/L, $P < 0.01$). Serum sIL-2R levels were significantly related to ALT levels. There was no significant difference in HBV DNA levels between the two groups (Table 1). Anti-HBc status was related to the age of the patients. Positive anti-HBc was detected in older patients ($P < 0.01$, Table 2).

DISCUSSION

HBV infection is a major health problem. About 350 million persons are chronically infected with HBV in the world. HBV itself is non-cytopathic and it is widely accepted that the mechanism of hepatocellular injury is the host anti-viral immune response^[9]. A human leukocyte antigen (HLA) class I-restricted cytotoxic T-lymphocyte (CTL) response to one or more HBV-encoded antigens on the hepatocyte membrane is a major mechanism of hepatocellular injury and clearance of infected cells^[10]. Serum sIL-2R is predominantly released from activated T lymphocytes^[11]. It was reported that serum sIL-2R levels reflect cellular IL-2 receptor expression^[6]. Hence, levels of serum sIL-2R are useful in monitoring T-lymphocyte activity and serial measurement aids in assessing the progression of the disease^[12].

High levels of serum sIL-2R have been observed in patients with chronic HBV infection^[7,8,12-14] and hepatitis C virus (HCV) infection^[15]. Serum sIL-2R levels indicate the degree of liver damage in patients with chronic HBV infection^[8]. Our results showed that serum sIL-2R levels were significantly higher in patients with chronic HBV infection than in healthy controls. The serum sIL-2R levels were significantly related to the serum ALT levels, but did not correlate with serum HBV DNA levels in patients with chronic HBV infection. These results are consistent with previous findings of Sawayama *et al*^[8].

Anti-HBc is detected in virtually all patients exposed

to HBV^[16] and typically persists for life^[17]. However, many patients with chronic HBV infection are negative for anti-HBc in China probably due to the fact that Chinese people acquire the infection at birth or during the early postnatal period^[18,19]. These patients may have an immune tolerance to the virus for several decades of life^[20,21]. We found that serum sIL-2R levels were significantly higher in patients with HBsAg (+), HBeAg (+) and anti-HBc (+) than in patients with HBsAg (+), HBeAg (+) and anti-HBc (-). Furthermore, patients with anti-HBc (+) were older than those with anti-HBc (-). Transfer of hepatitis B core antigen-reactive T cells is associated with the resolution of chronic HBV infection^[22]. Based on these results, it seems that patients with chronic HBV infection who are negative for anti-HBc may be in a status of immune tolerance to the virus. Serum sIL-2R levels and anti-HBc may be useful indicators of immune status in patients with chronic HBV infection. Serum sIL-2R levels reflect the activation of T lymphocytes^[5]. Hence, positive anti-HBc may be related to the activation of T lymphocytes.

Though interferon alpha to some extent hastens the loss of HBeAg in Chinese patients, the treatment is generally less effective than in white patients. This is probably due to the fact that the majority of Chinese people have a long period of immune tolerance to the virus^[23]. Several studies have revealed that serum sIL-2R levels can serve as an index of the activation of T lymphocytes^[5,6]. The serum sIL-2R level one year after interferon administration may be a useful marker of its therapeutic effectiveness^[8]. Our results showed that elevated serum sIL-2R and positive anti-HBc were related to the high levels of serum ALT in patients with chronic HBV infection. Low serum ALT levels are associated with the poor response to interferon alpha treatment in patients with chronic HBV infection^[23]. Hence, we can deduce that elevated serum sIL-2R levels, positive anti-HBc and high ALT concentrations may serve as indicators for interferon alpha treatment in Chinese patients with chronic HBV infection. However, further exploration is needed.

In conclusion, serum sIL-2R levels are related to anti-HBc and serum ALT concentrations, but not related to HBV DNA levels in patients with chronic HBV infection. Positive anti-HBc may be related to T-lymphocyte activation and negative anti-HBc may imply immune tolerance in these patients.

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Role of nitric oxide in Toll-like receptor 2 and 4 mRNA expression in liver of acute hemorrhagic necrotizing pancreatitis rats

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Abstract

AIM: To investigate the role of nitric oxide (NO) in Toll-like receptor 2 (TLR2)/4mRNA expression in livers of acute hemorrhagic necrotizing pancreatitis (AHNP) rats.

METHODS: One hundred and ten SD male rats were randomly divided into sham-operated group ($n=10$), AHNP group ($n=30$), chloroquine (CQ)-treated group ($n=30$) and L-Arg-treated group ($n=40$). TLR2/4mRNA expression in the liver of AHNP rats was measured by RT-PCR.

RESULTS: Expression of TLR2/4mRNA could be detected in the liver of AHNP rats in sham-operated group ($0.155E-5 \pm 0.230E-6$ and $0.115E-2 \pm 0.545E-4$), but was markedly increased at 3 h in AHNP group ($0.197E-2 \pm 0.114E-3$ and $0.175 \pm 0.349E-2$) peaking at 12 h ($0.294E-2 \pm 0.998E-4$ and $2.673 \pm 2.795E-2$, $P < 0.01$). Hepatic injuries were aggravated, TNF- α concentration in the liver was increased and NO concentration was decreased ($P < 0.05$ or $P < 0.01$). When TLR2/4mRNA expression was inhibited by CQ (3 h: $1.037E-4 \pm 3.299E-6$ and $0.026 \pm 3.462E-3$; 6 h: $1.884E-4 \pm 4.679E-6$ and $0.108 \pm 6.115E-3$; 12 h: $2.443E-4 \pm 7.714E-6$ and $0.348 \pm 6.807E-3$; $P < 0.01$), hepatic injuries were relieved, NO concentration in the liver was increased and TNF- α concentration was decreased ($P < 0.05$ or $P < 0.01$). When rats with AHNP

were treated with L-Arg, TLR2/4mRNA expression in the liver could be effectively inhibited (50 mg-T: $0.232E-2 \pm 0.532E-4$ and $0.230 \pm 6.883E-3$; 100 mg-T: $0.210E-2 \pm 1.691E-4$ and $0.187 \pm 0.849E-2$; 200 mg-T: $0.163E-2 \pm 0.404E-4$ and $0.107 \pm 0.195E-2$; 400 mg-T: $0.100E-2 \pm 0.317E-4$ and $0.084 \pm 0.552E-2$; $P < 0.01$) and hepatic injuries were relieved. At the same time, NO concentration in the liver was markedly increased and TNF- α concentration was decreased ($P < 0.05$ or $P < 0.01$).

CONCLUSION: The expression of TLR2/4mRNA is increased and hepatic injuries are aggravated in the liver of AHNP rats. TLR2/4mRNA gene expression in the liver of AHNP rats can be markedly inhibited by NO, leading to the relief of hepatic injuries.

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Key words: Toll-like receptors; Acute hemorrhage necrotizing pancreatitis; Liver; Nitric oxide; Chloroquine

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INTRODUCTION

Acute hemorrhagic necrotizing pancreatitis (AHNP) is a serious disease of human beings with a high mortality and morbidity. AHNP could cause multiple organ dysfunction syndrome (MODS). Unfortunately, the pathogenesis and mechanism of AHNP are still unclear. Many researches indicate that diverse inflammatory factors such as tumor necrosis factor (TNF- α), interleukin-1 (IL-1), IL-6 and reactive oxygen species result in systemic inflammatory response syndrome (SIRS) which might play an important role in the pathogenesis and development of AHNP^[1-3]. It was reported that Toll-like receptor 2 (TLR2)/4 activated by stimulations can result in excessive production and release of cytokines^[4]. In the present study, we have investigated the changes of TLR2/4 gene expression and the effect of NO on TLR2/4 gene expression in livers of AHNP rats.

Table 1 Serum amylase, ALT, and AST concentrations (mean±SD)

| | <i>n</i> | Serum amylase (U/L) | ALT (U/L) | AST (U/L) |
|--------|----------|---------------------------------|--------------------------|----------------------------|
| A unit | 10 | 985±159.68 | 74.0±4.47 | 176.6±4.52 |
| B unit | 10 | 6 367±1 122.17 ^b | 101.8±4.11 ^a | 447.9±54.49 ^b |
| C unit | 10 | 9 370±2 282.79 ^b | 232.9±24.01 ^b | 1055.9±41.57 ^b |
| D unit | 10 | 13 189±3 365.14 ^b | 546.5±37.36 ^b | 1276.2±44.22 ^b |
| E unit | 10 | 3 450±711.25 ^{bd} | 95.5±4.19 ^b | 370.3±19.67 ^b |
| F unit | 10 | 4 165±1 005.31 ^{b,c,e} | 119.7±4.74 ^{bd} | 784.6±68.93 ^{bd} |
| G unit | 10 | 5 540±1 274.81 ^{b,c,e} | 197.5±9.09 ^{bd} | 982.7±46.22 ^{bd} |
| H unit | 10 | 6 793±1 414.78 ^b | 212.8±5.50 ^b | 854.3±53.26 ^{bd} |
| I unit | 10 | 6 518±246.13 ^{bc} | 142.2±7.73 ^{bd} | 405.9±62.62 ^{bd} |
| J unit | 10 | 5 462±822.44 ^{bc} | 115.4±6.30 ^{bd} | 385.4±6.72 ^{bc,d} |
| K unit | 10 | 4 789±826.59 ^{bc} | 92.3±3.69 ^{bd} | 309.7±15.90 ^{bd} |

^a*P*<0.05, ^b*P*<0.01 vs A unit; ^c*P*>0.05 vs ahead unit; ^d*P*<0.05, ^e*P*<0.01 vs unit at the same time point of AHNP group.

MATERIALS AND METHODS

Chloroquine (CQ) and sodium taurocholate (TAC) were purchased from Sigma (St. Louis, MO, USA). L-Arg was purchased from Cayman Chemical Company, USA. Trizol was purchased from Promega Co., Hong Kong, China. Reverse transcriptase RNase and DNA polymerase were purchased from TOYOBO CO., LTD, Japan. Serum-amylase and NO detection kits were provided by Jiancheng Biological Engineering Research Institute, Nanjing, China.

Groups and models

One hundred and ten SD male rats (weighing 180-200 g) were purchased from Experimental Animal Center of Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China. Rats were randomized into sham-operated group (*n*=10), AHNP group (3, 6, and 12 h, units B-D, *n*=10), CQ-treated group (3, 6, and 12 h, units E-G, *n*=10), L-Arg-treated group (50, 100, 200, and 400 mg; units H-K; *n*=10).

AHNP was induced by infusion of 5% TAC (1 mL/kg) into biliopancreatic duct. After models of AHNP were made, L-Arg (100 mg/kg) was immediately injected via inferior vena to make L-Arg-treated models. Sham-operated models were made by flipping ceca. Samples of liver and blood were taken for analysis.

Alanine aminotransferase, aspartate aminotransferase, AST, serum amylase, and NO concentrations in the liver

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were detected to assess the degrees of hepatic injuries. ALT and AST concentrations were measured using an automatic biochemistry analyzer. Concentrations of serum amylase and NO in the liver were measured spectrophotometrically.

TLR2/4 mRNA and TNF-α mRNA gene expression in the liver

The expression of TLR2/4mRNA and TNF-αmRNA in the liver was assayed by RT-PCR. The sequence of TLR2 primer was 5'-CGCTTCCTGA ACTGTCC-3' (sense), 5'-GGTTGTCACCTGCTTCCA-3' (anti-

sense) and 5'-ACTAAGAGGCGGAGCGGA-3' (fluorescent probe). The sequence of TLR4 primer was 5'-ATCATGGCATTTGTTCTTCTTCT-3' (sense), 5'-CTGAGATTCTG ATCCATGCATTG-3' (anti-sense) and 5'-TCGGTAACG ACGTTGTAG-3' (fluorescent probe). The sequence of TNF-α primer was 5'-CCCGTCG GAACAGGGA ACTT-3' (sense), 5'-GGGTGTCCTTAGGGCAAG-3' (anti-sense) and 5'-CGAGGAGGCGAACCACCAA-3' (fluorescent probe). The sequence of β-actin primer 5'-GAACGGTGAAGGTGACAG-3' (sense), 5'-TAGA GAGAGTGGGGTGG-3' (anti-sense) and 5'-ACCACAGCACCTGCGG GAT-3' (fluorescent probe). Results were obtained using FTC-2000 real-time instrument (Fengling Biotechnology Limited Company, Shanghai, China).

Statistical analysis

The data were expressed as mean ± SD. The differences between the two groups were assessed by Student's *t*-test. *P*<0.05 was considered statistically significant.

RESULTS

Serum amylase, ALT and AST concentrations

Serum amylase concentration was lower in CQ-treated and L-Arg-treated groups than in AHNP group (*t*: 2.087-2.195, *P*<0.05). ALT and AST concentrations were low in sham-operated group but significantly increased in AHNP group (*t*: 4.58-9.740, *P*<0.01). Administration of CQ or L-Arg significantly reduced ALT and AST concentrations in the liver (*t*: 1.074-8.765, *P*<0.05, Table 1).

NO, TNF-α concentrations and TLR2/4mRNA expression

TNF-α was low in sham-operated group but significantly increased in AHNP group (*t*: 6.848-9.959, *P*<0.01). Administration of CQ or L-Arg significantly reduced TNF-α concentration in liver (*t*: 3.946-8.997, *P*<0.01). NO concentration was markedly lower in AHNP group than in sham-operated group (*t*: 2.403-8.521, *P*<0.05, CQ-treated group and L-Arg-treated group (*t*: 2.138-9.597, *P*<0.05). TLR2/4mRNA expression was low in sham-operated group but markedly increased at 3 h in AHNP group and peaked at 12 h (*t*: 2.193-9.623, *P*<0.01). TLR2/4mRNA expression was inhibited by CQ (*t*: 2.294-8.382, *P*<0.01), and L-Arg (*t*: 3.880-8.995, *P*<0.05; Table 2).

DISCUSSION

Ten members have been identified from the mammalian TLR family^[5,6]. TLRs belong to a wider superfamily, called IL-1 receptors/TLR superfamily, including receptors for the pro-inflammatory cytokines IL-1 and IL-18. All members possess cytoplasmic Toll/IL-1 receptor (TIR) domains. The TIR domain consisting of 160 amino acids is essential for signaling. TLRs are the key front-line sensors of invading microbes, responding to a wide range of microbial products through recognizing a different pathogen-associated molecular pattern (PAMP). At the same time, when TLRs are combined with PAMP, antigen presenting cells (APCs) are activated and produce co-

Table 2 NO and TNF- α concentrations, and TLR2/4mRNA expression in livers (mean \pm SD)

| | <i>n</i> | NO (μ mol/gprot) | TNF- α | TLR2 | TLR4 | |
|---|----------|-----------------------|------------------------------------|-------------------------------------|--|-------------------------------------|
| A | unit | 10 | 16.19 \pm 0.862 | 0.003 \pm 0.129E-3 | 0.115E-5 \pm 0.229E-6 | 0.1145E-2 \pm 0.545E-4 |
| B | unit | 10 | 12.91 \pm 1.058 ^a | 2.331 \pm 0.101 ^b | 0.197E-2 \pm 0.114E-3 ^a | 0.175 \pm 0.349E-2 ^b |
| C | unit | 10 | 9.53 \pm 0.344 ^b | 1.618 \pm 0.173 ^b | 0.275E-2 \pm 0.352E-4 ^b | 0.285 \pm 0.516E-2 ^b |
| D | unit | 10 | 4.52 \pm 0.356 ^b | 0.296 \pm 0.04 ^b | 0.294E-2 \pm 0.998E-4 ^{b,c} | 2.673 \pm 2.795E-2 ^b |
| E | unit | 10 | 27.78 \pm 0.542 ^{b,d} | 1.440 \pm 5.147E-2 ^{b,d} | 1.037E-4 \pm 3.299E-6 ^{b,d} | 0.026 \pm 3.462E-3 ^{b,d} |
| F | unit | 10 | 20.73 \pm 0.462 ^{b,d} | 0.862 \pm 3.197E-2 ^{b,d} | 1.884E-4 \pm 4.679E-6 ^{b,d} | 0.108 \pm 6.115E-3 ^{b,d} |
| G | unit | 10 | 13.70 \pm 0.734 ^{a,d} | 0.117 \pm 1.492E-2 ^{b,d} | 2.443E-4 \pm 7.714E-6 ^{b,d} | 0.348 \pm 6.807E-3 ^{b,d} |
| H | unit | 10 | 9.87 \pm 0.095 ^b | 0.676 \pm 2.092E-2 ^{b,d} | 2.324E-2 \pm 0.532E-4 ^{b,d} | 0.229 \pm 6.883E-3 ^{b,d} |
| I | unit | 10 | 11.69 \pm 0.954 ^{b,c,d} | 0.369 \pm 1.300E-2 ^{b,d} | 0.210E-2 \pm 1.691E-4 ^{b,c,d} | 0.187 \pm 0.085E-3 ^{b,d} |
| J | unit | 10 | 25.43 \pm 0.919 ^{b,d} | 0.115 \pm 0.587E-2 ^{b,d} | 0.163E-2 \pm 0.404E-4 ^{b,e} | 0.107 \pm 1.946E-3 ^{b,d} |
| K | unit | 10 | 32.99 \pm 0.382 ^{b,d} | 0.058 \pm 0.652E-2 ^{b,d} | 0.100E-2 \pm 0.316E-4 ^{b,e} | 0.084 \pm 5.522E-3 ^{b,d} |

^a P <0.05, ^b P <0.01 vs A unit; ^c P >0.05 vs ahead unit; ^d P <0.01, ^e P <0.05 vs unit at the same time point of AHNP group.

stimulatory molecules and cytokines, activating specific immune responses. TLR2/4 plays the most important role in responding to bacterial infections. TLR2 is the major receptor for PAMPs of Gram-positive bacteria, such as peptidoglycan and lipoteichoic acids. TLR4, the major receptor for LPS, are highly susceptible to infections with Gram-negative bacteria and fungal pathogens, such as lipopolysaccharide (LPS) and heat shock protein (HSP)^[7]. Mechanisms responsible for TLR-mediated protection, potentiation of cytokine release, mediation of neutrophil recruitment to the site of infection and release of oxygen and nitrogen radicals, and contribute to TLR activation^[8]. Researches showed that TLR2^{-/-} or TLR4^{-/-} mice have an increased susceptibility to infections^[9-12]. NO produced from L-Arg by catalysis of nitric oxide synthase (NOS) is the only resource of NO in body^[13]. In our experiment, rats were injected with L-Arg so that NO concentration in the bodies of rats was elevated. At the same time, it was reported that TLR2/4mRNA expression can be inhibited by CQ^[14]. In the present study, we investigated the changes of TLR2/4 gene expression and NO concentration in the liver when TLR2/4mRNA expression was inhibited by CQ and the effect of NO on TLR2/4 gene expression in the liver of AHNP rats.

Enteric bacteria and endotoxin can result in aggravation of AHNP. In the course, enteric bacteria and endotoxin enter the liver through portal vein and are deactivated. But when the function of the liver is damaged or the quantity of enteric bacteria and endotoxin exceeds the endurance of liver, bacteria and endotoxin enter the blood and result in sepsis. At the same time, AHNP can lead to the dysfunction of the liver and even the failure of liver, suggesting that liver may play an important role in the pathogenesis and development of AHNP.

Our study showed that TLR2/4mRNA expression could be detected in sham-operated group (0.155E-5 \pm 0.230E-6 and 0.115E-2 \pm 0.545E-4), but markedly increased at 3 h in AHNP group (0.197E-2 \pm 0.114E-3 and 0.175 \pm 0.349E-2) and peaked at 12 h (0.294E-2 \pm 0.998E-4 and 2.673 \pm 2.795E-2, P <0.01). Hepatic injuries were aggravated, while TNF- α concentration increased and NO concentration decreased (P <0.05). When TLR2/4mRNA expression was inhibited

by CQ (3 h: 1.037E-4 \pm 3.299E-6 and 0.026 \pm 3.462E-3; 6 h: 1.884E-4 \pm 4.679E-6 and 0.108 \pm 6.115E-3; 12 h: 2.443E-4 \pm 7.714E-6 and 0.348 \pm 6.807E-3; P <0.01), hepatic injuries were relieved while NO concentration increased and TNF- α concentration decreased (P <0.05). When AHNP rats were treated with L-Arg, TLR2/4 mRNA expression was effectively inhibited (50 mg-T: 0.232E-2 \pm 0.532E-4 and 0.230 \pm 6.883E-3; 100 mg-T: 0.210E-2 \pm 1.691E-4 and 0.187 \pm 0.849E-2; 200 mg-T: 0.163E-2 \pm 0.404E-4 and 0.107 \pm 0.195E-2; 400 mg-T: 0.100E-2 \pm 0.317E-4 and 0.084 \pm 0.552E-2; P <0.01) and hepatic injuries were relieved. At the same time, NO concentration markedly increased and TNF- α concentration decreased (P <0.05).

When TLR2/4mRNA expression inhibited, synthesis and release of inflammatory factors decreased and hepatic injuries were relieved, suggesting that lower concentration of NO has the anti-inflammatory effect^[15,16]. In our study, NO concentration in AHNP rats was decreased. When TLR2/4mRNA expression was inhibited by CQ, NO concentration increased, suggesting that TLR2/4mRNA expressions can be inhibited by NO. Our results suggest that synthesis and release of anti-inflammatory factors might be inhibited by TLR2/4mRNA expression and TLR2/4mRNA gene expression might play an important role in the pathogenesis and development of hepatic injury in AHNP.

Lower concentration of NO may exert its anti-inflammatory effect by reducing neutrophilic leukocytes, platelet and adhesion molecules, concentration of inflammatory factors in bronchoalveolar lavage, improving the pancreatic blood flow and pancreatic microcirculation, inhibiting the production of oxyradicals and cytokines, interaction of leukocytes and endotheliocytes.

NO may decrease TLR2/4mRNA expression by directly inhibiting production of cytokines, reducing differentiation of phagocytes and interaction between leukocytes and endotheliocytes. The results of our experiment provide evidence for the role of NO in TLR2/4mRNA expression in the liver of AHNP rats.

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Retention mucocele of distal viable remnant tip of appendix: An unusually rare late surgical complication following incomplete appendectomy

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Abstract

A 67-year old man was presented with a 6-mo history of recurrent right lower quadrant abdominal pain. On physical examination, a vague mass was palpable in the right lumbar region. His routine laboratory tests were normal. Ultrasonography showed a hypoechoic lesion in the right lumbar region anterior to the right kidney with internal echoes and fluid components. Abdominal contrast-enhanced computed tomography (CECT) showed a well-defined hypodense cystic mass lesion lateral to the ascending colon/caecum, not communicating with the lumen of colon/caecum. After complete open excision of the cystic mass lesion, gross pathologic examination revealed a turgid cystic dilatation of appendiceal remnant filled with the mucinous material. On histopathological examination, mucinous cyst adenoma of appendix was confirmed. We report this rare unusual late complication of mucocele formation in the distal viable appendiceal remnant, which was leftover following incomplete retrograde appendectomy. This unusual complication is not described in the literature and we report it in order to highlight the fact that a high index of clinical and radiological suspicion is essential for the diagnosis of mucocele arising from a distal viable appendiceal remnant in a patient who has already undergone appendectomy presenting with recurrent abdominal pain.

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Key words: Retention mucocele; Appendix; Incomplete appendectomy; Surgical complication

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INTRODUCTION

Appendiceal mucocele was first described by Rokitanski in 1842^[1] and an unusual variant of appendiceal mucocele -myxoglobulosis was first described by Latham in 1897^[1-3]. The term "mucocele of appendix"^[4,5] is an inherently imprecise descriptive term that refers to any macroscopic (localised or diffuse) globular cystic dilatation of appendix (unilocular or multilocular) filled with thick tenacious mucoid/mucinous material regardless of underlying cause and is not a pathologic entity.

The majority of mucoceles of appendix arise secondary to proximal obstruction of appendiceal lumen in which the appendiceal lumen is usually in communication with caecum^[6]. The potential causes of proximal appendiceal obstruction include faecolith, epithelial/mucosal hyperplasia, post inflammatory-fibrosis, cystadenoma, cystadenocarcinoma, carcinoid tumor, endometriosis and developmental anomalies such as occlusive membrane or obstructive diaphragm at the level of appendiceal orifice^[7]. But the origin of mucocele of appendix from a distal viable leftover remnant tip of appendix not communicating with the caecum following incomplete retrograde open appendectomy has not been described.

We report such an extremely rare case of retention mucocele arising from a distal vascularized remnant of appendix tip, which is a leftover following incomplete open appendectomy.

CASE REPORT

We report a 67-year old man who was presented with a 6-mo history of recurrent right lower quadrant abdominal pain not associated with vomiting. He had normal appetite and bowel habits. He was not a hypertensive and diabetic, neither an alcoholic nor a smoker. He underwent open appendectomy 15 years ago. At presentation his vital

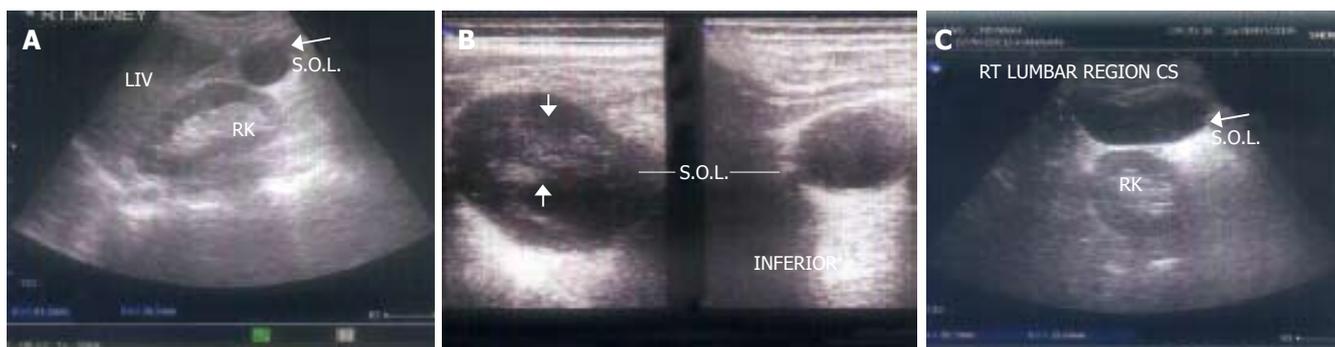


Figure 1 Ultrasonography shows a hypoechoic space occupying lesion measuring 6.5 cm × 3 cm × 6 cm in the right lumbar region anterior to right kidney (arrows). Internal echoes and fluid components are seen within the cystic mass (arrowheads) (A-C).

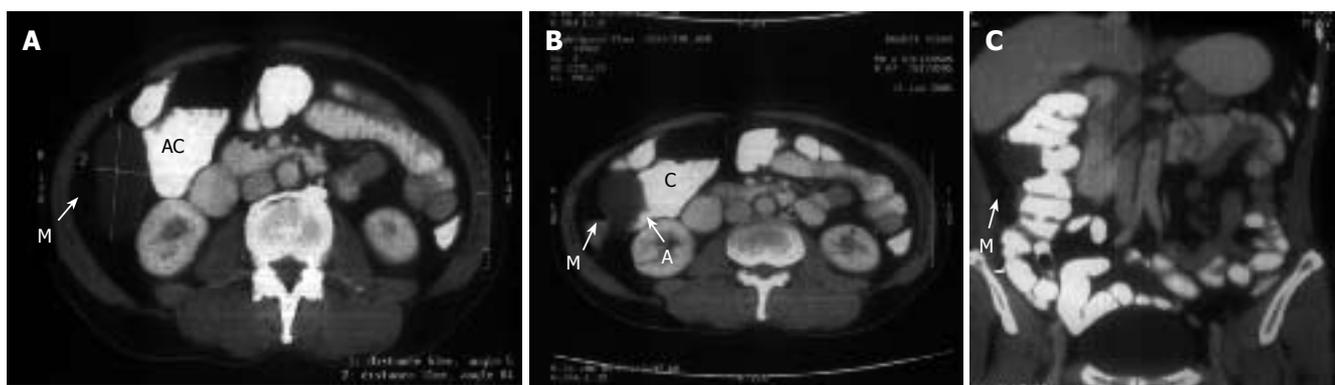


Figure 2 CECT shows a well defined hypodense blind ending tubular lesion (arrows) measuring 6.8cm × 3.1cm × 3.0cm (CT value +32HU) in the right lumbar region lateral to ascending colon along the antimesenteric border not communicating with the lumen of colon (A-C). C=Caecum, AC=Ascending colon, M=Mucocele, A=Distal appendiceal remnant not communicating with caecal lumen.



Figure 3 Macroscopic/gross pathology. **A:** Perioperative photograph shows a turgid cystic mass measuring 6cm × 5cm × 3cm in the distal appendicular remnant with a separate mesentery located adjacent to the pulled up caecum and not communicating with lumen of caecum (arrows); **B:** Resected specimen shows cystic dilatation of turgid distal appendicular remnant with surrounding fibrofatty, mesenteric, and omental tissues (arrow); **C:** Cut open resected specimen shows protruding mucinous material from the lumen of cystically dilated turgid distal appendicular remnant (arrow).

parameters and systemic examination were normal. On examination of abdomen, a vague mass was palpable in the right lumbar region suspicious of a retroperitoneal tumor. Rectal examination was normal.

Hemoglobin value was 13.8 gm/dL (reference range: 13.5-17.0 gm/dL) and other biochemical investigations, chest X-ray and electro-cardiogram were normal. Upper gastrointestinal (GI) endoscopy and colonoscopy study was normal. Ultrasonography revealed a 6.5cm × 3cm × 3.5cm hypoechoic space-occupying lesion in the right lumbar

region anterior to the right kidney with internal echoes and fluid components which suggested retroperitoneal cyst (Figure 1). Abdominal CECT showed a well defined blind ending tubular hypodense lesion with CT value +32 Hounsfield unit (HU) in the right lumbar region lateral to the ascending colon along the antimesenteric border (Figure 2). The lesion was not communicating with lumen of the colon, which suggested colonic duplication cyst.

At laparotomy, a 6cm × 3cm mucus-filled turgid mass was found in the distal appendiceal remnant having a

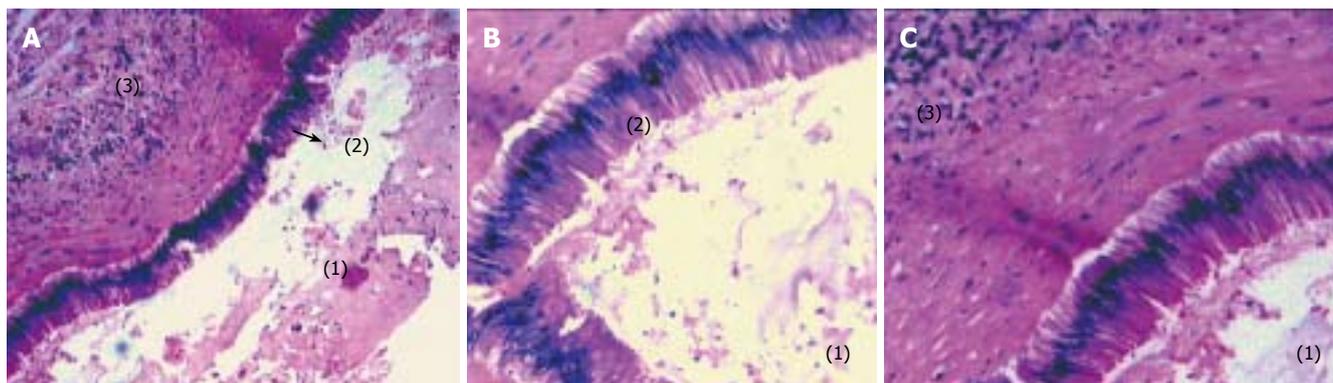


Figure 4 Histopathological microphotographs of mucinous cystadenoma of appendix. **A:** Low power microscopic view. Appendicular lumen shows mucinous secretions (1) with no epithelial elements. Mucosa lined by a single layer of mucinous columnar epithelium with basally situated nuclei (2) does not show any atypia with underlying lymphoid aggregates (3). **B,C:** High power microscopic view. Appendiceal lumen shows lakes of mucin (1) with no epithelial elements. Mucosa lined by a single layer of mucinous columnar epithelium with basally situated nuclei (2) does not show any atypia with underlying lymphoid aggregates (3).

separate mesentery with no communication with the caecum (Figure 3A). Complete surgical excision was performed with an uneventful recovery. Gross pathological examination showed a distended turgid appendiceal remnant filled with characteristic mucinous material (Figures 3B, 3C). Histopathological examination showed that the appendix was lined by a single layer of mucinous epithelium with basally situated nuclei which did not show any atypia with underlying lymphoid aggregates. The appendiceal lumen showed mucinous secretions (lakes of mucin) with no epithelial elements suggestive of mucinous cyst adenoma-appendix with no evidence of malignancy or dysplasia. (Figures 4A,4B,4C). The patient was symptom-free during a 6-mo follow-up period.

DISCUSSION

Primary appendiceal mucocele, a relatively uncommon clinical entity, is most frequently an incidental finding at the time of surgery and is occasionally discovered only at pathological examination^[8]. The majority of these patients are not diagnosed preoperatively and in fact 60%^[8] of them are diagnosed incidentally during surgery for some other disease. The incidence of appendiceal mucocele is estimated to be 0.2-0.3% of appendicectomy specimens with myxoglobulosis constituting 0.35-0.8% of mucoceles^[7].

Mucoceles are histologically subdivided into four types on the basis of World Health Organization classification^[4,8].

Simple/non neoplastic mucocele or retention mucocele or obstructive form of mucocele^[5] is defined as cystic dilatation of the distal appendix with accumulation of abnormal mucoid material in the appendiceal lumen secondary to appendiceal outflow obstruction.

Benign neoplastic mucocele-mucinous cystadenoma^[5] is defined as dilated mucus /mucin filled appendix containing adenomatous mucosa lined by atypical mucinous epithelium containing basal nuclei and showing only minimal dysplastic features. Secondary changes^[9] in mucinous cyst adenoma include thinning of the wall, extensive ulceration, calcification and ossification (“porcelain appendix”)^[5]. There is also a high association

between appendiceal mucinous cystadenoma with ovarian mucinous cyst adenoma and synchronous or metachronous neoplasms elsewhere in the colon^[10].

Malignant mucocele^[5]-mucinous cystadenocarcinoma is defined as adenocarcinoma associated with mucus-filled cystic dilatation of the appendix presenting as mucocele. A malignancy is suspected at surgery in about 30%^[4,5]. In the others the diagnosis is made during pathologic examination.

Cystadenocarcinoma^[4] is grossly indistinguishable from a cystadenoma. But histologically^[5] the former is distinguished from mucinous cystadenoma by three criteria: presence of invasive neoplasm below the level of muscularis mucosa, when the muscularis mucosa cannot be distinguished because of distortion or fibrosis, the diagnosis is made by the finding of infiltrative tongues of tumor or single tumor cells in the wall (infiltrative appearance of border of epithelial elements) as opposed to the broad pushing edge appearance of the borders of epithelial elements that characterize mucinous tumor of uncertain/undetermined malignant potential (UMP)^[5,11], presence of malignant epithelial cells in the lakes of mucin either in the wall of the appendix or outside the appendix.

Myxoglobulosis or Caviar appendix^[5,7] is an extremely rare variant of appendiceal mucocele caused by proximal obstruction of appendiceal lumen in which pieces of mucinous/mucoid material can become broken off the appendiceal wall into the appendiceal lumen resulting in the formation of characteristic pearl-like translucent “mucinous globules” or pearly luminal spheroids^[7] or a “cluster of frog eggs”^[12] 1-10 mm in diameter with surface calcification.

Mucocele of appendix is most common in the sixth or seventh decade of life with a female preponderance^[7]. The common presenting symptoms of appendiceal mucocele are episodic right lower quadrant abdominal pain (27%)^[8], abdominal mass (16%)^[8], weight loss (10%)^[8] and change in bowel habits (5%)^[8]. Complications^[12-15] of mucocele include intussusception, bleeding, perforation, peritonitis, rupture and pseudomyxoma peritonei.

Colonoscopy may show a smooth glassy submucosal

or extra-mucosal caecal mass moving in and out with respiratory movement. This endoscopic sign has been described as the "trapped balloon sign"^[14]. The classical CT scan findings^[7, 15] of a mucocele in a patient who has not undergone appendectomy are a cystic low attenuation well encapsulated round or ovoid mass with smooth regular walls in the right lower quadrant adherent to caecum, mural calcification in the wall of the mucocele, and absence of peri-appendiceal inflammation or abscess which is the key differentiating point in excluding acute appendicitis. The presence of thickened wall and enhanced nodules favors the diagnosis of mucinous cystadenocarcinoma.

The clinical and radiological differential diagnosis^[7, 13] of mucocele of appendix includes mesenteric cyst, colonic duplication cyst, colonic lymphoma and lipoma, intussusception, right ovarian cyst and hydrosalpinx.

The treatment of mucocele of appendix is essentially by simple appendectomy but in cases of rupture or suspected malignancy a standard right hemicolectomy is indicated^[8]. At the time of surgery, a spontaneous appendiceal perforation or any extravasation from appendicular lumen is strongly suggestive of malignancy in such situations, a right hemicolectomy^[8, 16] should always be performed with a curative intent. Laparoscopic appendectomy for mucocele removal has been described, but caution has also been suggested because of the risk of port site recurrences^[17].

In a patient who has undergone open appendectomy, with the CT finding of a cystic well encapsulated mass in the right lumbar region adherent to the pulled up caecum/ascending colon not communicating with the lumen of colon/caecum, one should consider the possibility of mucocele of distal appendiceal remnant. Therefore, a high index of clinical and radiological suspicion is essential for the preoperative diagnosis of mucocele of distal appendiceal remnant in a patient who has undergone appendectomy. Incomplete surgical removal of appendix must be avoided in order to prevent the late complication of mucocele formation in the distal leftover vascularized remnant tip of appendix. All appendiceal mucoceles measuring at least 2 cm must be completely excised to eliminate the chance of progression to malignancy. The association between appendiceal mucocele and colonic neoplasm is more clear and logical to recommend

surveillance colonoscopy in patients with diagnosis of appendiceal mucocele, at least in those with appendiceal mucinous cystadenoma.

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TIPSS for variceal hemorrhage after living related liver transplantation: A dangerous indication

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Abstract

The introduction of transjugular intrahepatic portal-systemic stent-shunt (TIPSS) has been a major breakthrough in the treatment of portal hypertension, which has evolved to a large extent, into a routine procedure. A 21-year-old male patient with progressive graft fibrosis/cirrhosis requiring TIPSS for variceal hemorrhage in the esophagus due to portal hypertension was unresponsive to conventional measures two years after living related liver transplantation (LDLT). Subsequently, variceal hemorrhage was controlled, however, liver function decreased dramatically with consecutive multi organ failure. CT scan revealed substantial necrosis in the liver. The patient underwent successful "high urgent" cadaveric liver transplantation and was discharged on postoperative d 20 in a stable condition.

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Key words: Portal hypertension; Liver necrosis; Fibrosis; Cirrhosis

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INTRODUCTION

Transjugular intrahepatic portal-systemic stent-shunt

(TIPSS) is defined as artificial shunts between branches of the portal vein and the systemic circulation within the liver parenchyma, and is comparable to a surgical H-shunt with partial decompression of portal hypertension. The first TIPSS has been performed by Rösch *et al*^[1] in 1969 in dogs. After the stage had been set for the implantation of stent-shunts into human beings, the first TIPSS was performed on a 49-year-old male patient with liver cirrhosis and bleeding from esophageal varices due to portal hypertension^[2]. Since then this procedure has rapidly progressed to a well defined and established standard procedure for a wide variety of liver disorders, i.e., TIPSS is preferred for acute or recurrent variceal hemorrhage refractory to medical treatment, endoscopic sclerotherapy or banding^[3]. Since TIPSS enables pre-transplant patients to recover from bleeding episodes and improves their general status before a graft becomes available, this procedure forms a bridge to liver transplantation^[4]. The first 30-d mortality after TIPSS (up to 11%) is usually due to liver failure, acute respiratory distress syndrome, sepsis, recurrent hemorrhage, or right heart or multi organ failure^[4]. Here we report a case of liver necrosis with subsequent organ failure making it necessary for high urgency (HU) cadaveric liver transplantation following TIPSS for conventionally uncontrolled variceal hemorrhage after living related liver transplantation (LDLT).

CASE REPORT

A 21-year-old male patient underwent LDLT with biliodigestive anastomosis because of liver cirrhosis due to hereditary tyrosinemia type I and hepatocellular carcinoma (T₃N₀M₀). After an uneventful transplantation, the postoperative course was further complicated by a bilioma followed by both a biliocutaneous fistula and chronic cholangitis. Two years after LDLT, the patient was referred to our institution and presented with progressive cholestasis, all signs of portal hypertension (i.e., varicose veins of the esophagus and hypersplenism) while graft function was still adequate, classified as Child-Pugh A. Post-contrast sequences of MRI originally performed to identify the total extent of the biliocutaneous fistula, showed perfusion of the liver as inhomogeneous with regular appearance of the intra- and extrahepatic portal venous branches, inferior vena cava (IVC) and hepatic artery (Figure 1A). For a complete

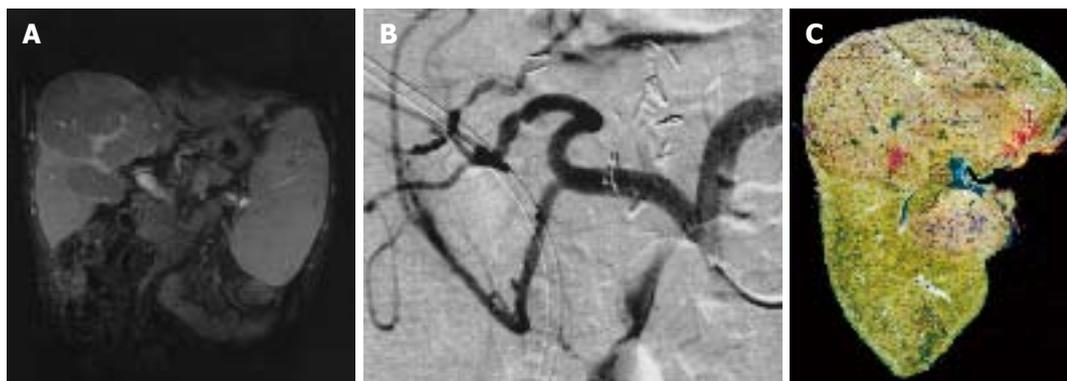


Figure 1 Pre-TIPSS MRI/angiography and post-TIPSS macroscopy of the liver. Pictures illustrating pre-TIPSS inhomogeneous intrahepatic parenchyma texture (A); clinically irrelevant stenosis of the hepatic artery (B) and macroscopic aspect of the liver after TIPSS and organ failure due to substantial necrosis in areas where circulation was compromised before TIPSS (C).

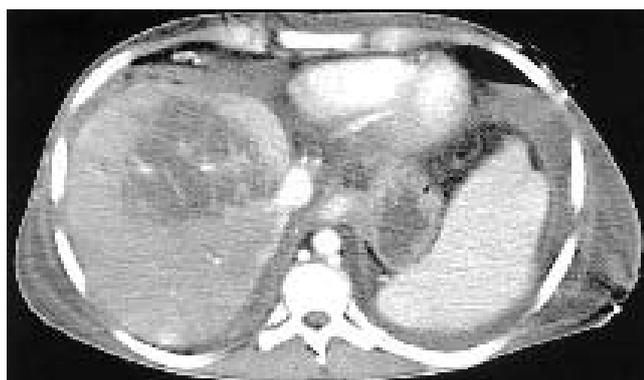


Figure 2 TIPSS-placement. Picture illustrating correctly placed TIPSS.



Figure 3 Spiral-CT scan after TIPSS was established. Spiral-CT after TIPSS revealed substantial necrosis at the time of liver failure.

examination of the patient's preoperative status, a celiac-mesenteric angiography was performed which identified a stenosis of the arterial anastomosis, considered as hemodynamically irrelevant (Figure 1B). Revision of the biliodigestive anastomosis was performed and a y-Roux hepatojejunostomy after adhesiolysis was established to close the biliocutaneous fistula, which maintained cholangitis. Histology of the intraoperative biopsy revealed severe fibrosis associated with secondary biliary cirrhosis of the transplant. After surgery the patient recovered quickly; however, on postoperative d 10 recurrent variceal hemorrhage unresponsive to conventional measures, including endoscopic therapy, required TIPSS since surgery was not feasible at that time. TIPSS was performed in a standard procedure with an uncoated 8/39 Corinthian-Stent, which decreased the portacaval pressure gradient from 23 mmHg (measured after a 8 mm tract was established) to 8 mmHg at the end of the procedure (Figure 2). Subsequently, variceal hemorrhage in the esophagus stopped, however, serum transaminases, bilirubin and ammonia increased, while liver function decreased dramatically with fulminant consecutive multi organ failure within 3 postoperative days. At that time, a spiral-CT scan revealed both substantial disturbances of intrahepatic perfusion and necrosis, while TIPSS, extrahepatic portal vein, hepatic artery and IVC displayed normal (Figures 2 and 3). One day later, the patient underwent successful (HU) orthotopic cadaveric re-transplantation of the liver and was discharged in a stable condition thereafter on postoperative d 20.

DISCUSSION

Biliary complications after LDLT are observed in 15-40% of patients, which can increase the risk of developing secondary biliary cirrhosis and all signs of portal hypertension (i.e. variceal bleeding)^[5]. The significant decrease of portal flow to the liver after TIPSS, due to portal decompression, may result in decreased liver function. An increase in hepatic arterial flow, however, maintains sufficient liver function in most patients^[9]. The latter is inadequate in a small number of patients and TIPSS results in liver failure^[4]. Further, cirrhotic patients have a cirrhotic cardiomyopathy. This is important since TIPSS shunts a large blood volume back to the right heart and may impair cardiac function.

Till date most information of both arterial and portal venous hemodynamic in the liver are based on indirect scintigraphic or invasive electromagnetic measurement of blood flow during portosystemic shunt surgery. Burchell *et al*^[6] demonstrated in their early work an increased blood flow in the hepatic artery after porto-caval shunting. Since no flow measurements were performed intravascularly, precise information on complex flow changes over time (i.e., detection of mechanisms of arterio-portal compensation) were impossible^[6]. In the late sixties Hanson^[7] and Lutz^[8] hypothesized that both dilatation and contraction of the intrahepatic arterioles, which occur immediately after liver- or shunt-operations, can be defined as regulatory reflex-mediated responses to changes of pressure in the hepatic sinusoids. Richter *et al*^[9] later demonstrated

online the dynamic changes of arterial and portal blood flow during partial porto-systemic decompression with TIPSS. Real-time monitoring of the arterial flow with endoluminal catheters revealed that arterial blood flow increased significantly and momentarily while portal venous flow decreased; however, total liver perfusion after TIPSS maintained steady in their studies. Since the fraction of arterial perfusion was increased after TIPSS, both pressure of portal venous perfusion and portal flow in hepatic sinusoids decreased. Reduced portal venous inflow in hepatic capillaries was compensated by an increased arterial perfusion. Since these changes in hepatic flow were immediately present in all the patients after TIPSS, underlying regulatory mechanisms that maintain a constant sinusoidal liver perfusion are most likely based on reflexes.

How can it be explained that major disturbances in hepatic circulation with subsequent liver necrosis and deterioration of liver function occurred after TIPSS in the case described above? Several factors most likely contributed to pathology: One major factor was the initially compensated stenosis of the hepatic artery, diagnosed with a celiac-mesenteric angiography (Figure 1B), which most likely prevented an increase in the arterial flow reserve to compensate for a decreased portal flow after TIPSS. Moreover, chronic inflammation (cholangitis) and liver cirrhosis may have also factored into the reduction of possible regulatory mechanisms stated above that compensated for reduced portal flow^[10,11]. Therefore, severe dysfunction of the liver due to disturbances in hepatic perfusion, with subsequent necrosis of liver tissue, complicated treatment after TIPSS (Figures 1C and 3). In such cases, TIPSS should be indicated with great caution in centers where the salvage therapy, high urgent liver transplantation, can be performed.

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CASE REPORT

Extensive retroperitoneal and right thigh abscess in a patient with ruptured retrocecal appendicitis: An extremely fulminant form of a common disease

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Abstract

As a disease commonly encountered in daily practice, acute appendicitis is usually diagnosed and managed easily with a low mortality and morbidity rate. However, acute appendicitis may occasionally become extraordinarily complicated and life threatening. A 56-year-old man, healthy prior to this admission, was brought to the hospital due to spiking high fever, poor appetite, dysuria, progressive right flank and painful swelling of the thigh for 3 d. Significant inflammatory change of soft tissue was noted, involving the entire right trunk from the subcostal margin to the knee joint. Painful disability of the right lower extremity and apparent signs of peritonitis at the right lower abdomen were disclosed. Laboratory results revealed leukocytosis and an elevated C-reactive protein level. Abdominal CT revealed several communicated gas-containing abscesses at the right retroperitoneal region with mass effect, pushing the duodenum and the pancreatic head upward, compressing and encasing inferior vena cava, destroying psoas muscle and dissecting downward into the right thigh. Laparotomy and right thigh exploration were performed immediately and about 500 mL of frank pus was drained. A ruptured retrocecal appendix was the cause of the abscess. The patient fully recovered at the end of the third post-operation week. This case reminds us that acute appendicitis should be treated carefully on an emergency basis to avoid serious complications. CT scan is the diagnostic tool of choice, with rapid evaluation followed by adequate drainage as the key to the survival of the patient.

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Key words: Acute appendicitis; Retrocecal appendicitis; Complication; Retroperitoneal abscess; Thigh abscess

INTRODUCTION

Acute appendicitis is a disease commonly encountered in daily practice, and a very low morbidity and mortality rate can be achieved with proper diagnosis and management at present^[1,2]. Generally, a non-perforated acute appendicitis can be managed by urgent appendectomy, while perforated appendicitis which may be associated with the formation of localized abscess in the right iliac fossa or in the pelvic cavity, can be managed depending on their symptoms either by early appendectomy or by interval appendectomy following percutaneous drainage^[3]. Even with a more severe form of ruptured appendicitis such as those complicated with diffuse peritonitis as commonly encountered in patients of pre-school age, the post-operative recovery is usually smooth^[4]. However, acute appendicitis such as those forming appendiceal masses and extensive abscesses or resulting in intestinal obstruction may sometimes become more complicated and require a prolonged treatment period^[3,4]. These complications should not be overlooked in order to avoid further sequelae. Hence, we present here a rare and critical case of ruptured retrocecal acute appendicitis with extensive formation of retroperitoneal and thigh abscess, re-emphasizing the importance of early diagnosis and prompt management for this common disease.

CASE REPORT

A 56-year-old man was generally in good health when he first experienced right flank and right thigh pain with daily spiking fever of up to 39°C for 3 d. He also experienced progressive loss of appetite, epigastric fullness and painful disability of the right thigh. There was minimal abdominal pain initially, but this became more prominent over the right lower quadrant a few days later. He was brought to our hospital 3 d after the onset of the symptoms.

Physical examination on admission revealed an acute ill-

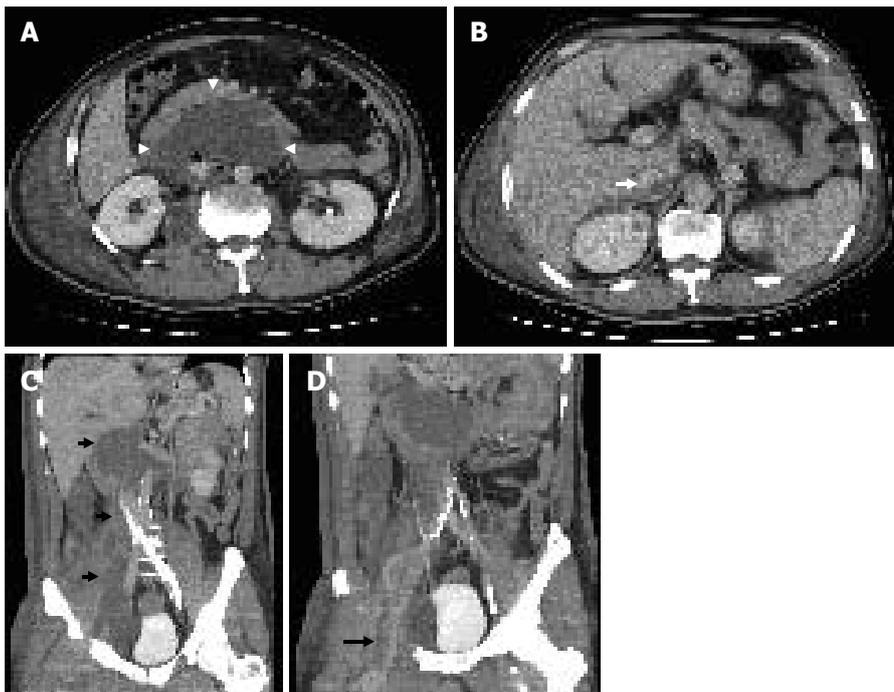


Figure 1 Retroperitoneal and right thigh abscess in a patient with ruptured retrocecal appendicitis. **A:** Retroperitoneal abscess with mass effect, demonstrating the upward-pushed duodenum and pancreatic head as well as compressed and encased inferior vena cava (white arrowheads); **B:** suspicious septic thrombus inside inferior vena cava (white arrow); **C:** reconstructed coronal image demonstrating extensive involvement of the abscess (short black arrows); **D:** dissection of the abscess in the right thigh via the femoral canal just beneath the femoral vessels (long black arrow).

looking man with a body temperature of 39.6°C. He was slightly anemic in appearance and breathed both shallowly and rapidly at a rate of 28/min. He was lying in supine position with his right knee joint mildly flexed and hip joint externally rotated, being reluctant to move his right leg because of severe tenderness. A positive psoas stretch test was performed and the results indicated that there were significant inflammatory signs such as local heat, swelling, edema, and tenderness disclosed at areas involving the entire right trunk from right subcostal region to right knee joint, but no subcutaneous emphysema or crepitation was noted. Palpation of the abdomen revealed tenderness at right lower quadrant without muscle rigidity and no mass-like lesion was palpable. Laboratory data indicated leukocytosis with a WBC count of $16.4 \times 10^6/\text{mL}$, of which 80% were mature neutrophils and 3% were immature neutrophils. The hemoglobin level was slightly decreased to the level of 12.8 g/dL and his platelet count was also decreased to $617 \times 10^6/\text{mL}$. The C-reactive protein level was 26.1 mg/dL. All the other blood chemistry data were within the normal range. X-ray of the abdomen revealed an indistinct shadow of right psoas muscle. CT scan of the abdomen revealed formation of multiple gas-containing abscesses involving the entire right retroperitoneum with mass effect. At the upper abdominal region, the duodenum and pancreatic head were pushed upward by the abscess, the right perinephric space was filled with the abscess and the inferior vena cava at the same level was encased and compressed. There was also a suspicious septic thrombus inside the inferior vena cava. In addition to destroying the right psoas muscle, the abscess also dissected downward to the right thigh through the femoral canal, forming an abscess between muscle groups (Figure 1).

The patient underwent a laparotomy immediately for retroperitoneal exploration, which revealed more than 500 mL of feculent fluid collection in the above-mentioned locations. However, the intra-abdominal cavity

was clear without any contamination. A gangrenous ruptured appendix was identified with its entire length embedded in the retroperitoneum, draining fecal content into it and forming an abscess. Appendectomy was done cautiously making sure that no necrotic residual appendix remained in the retroperitoneal cavity. The abscess was communicated with those at the thigh as expected, through the femoral canal just behind the inguinal ligament, even though a separate incision was made at the right thigh to ensure complete drainage of the abscess. Fortunately, the muscle groups of the right thigh were still viable and therefore no debridement of the muscle was performed. Multiple sump drainages were inserted at the end of the operation. The bacterial culture revealed an *Escherichia coli* infection.

The patient recovered smoothly with his fever subsiding 2 d after surgery and oral intake resumed in 4 d. Painful disability of his right thigh improved immediately after surgery and he was able to walk 12 d later. The patient was discharged uneventfully 3 wk after the surgery.

DISCUSSION

Acute appendicitis is the most common abdominal emergency worldwide and can usually be managed smoothly even if it is perforated. However, formation of retroperitoneal abscesses remains one of the most serious but rare complications of acute appendicitis and is always associated with perforation of a retrocecal appendix due to delayed diagnosis and treatment^[5-7]. Knowing that the anatomical position of the appendix is variable and 65% of the appendix has been reported to be at the retrocecal^[8], the importance of early management for acute appendicitis cannot be over-emphasized.

There are quite a few case reports discussing related problems similar to the present case, such as psoas and thigh abscesses^[9,10], lower extremity subcutaneous

emphysema of abdominal origin^[11-15] and rare complications of acute appendicitis^[16,17]. Among these reports, descriptions are similar regarding patients' presentation and their management. In short, the onset of symptoms is usually insidious and atypical, initial medical treatment is usually unsuccessful. The causes of abscess formation are usually unclear before surgery and patients are usually critical on presentation. Surgical management is mandatory but may or may not be effective and mortality is not an uncommon result. In addition to the above-mentioned experiences, there are still some important issues that can be addressed based on our present case and a literature review.

First of all, it is still difficult to distinguish primary from secondary psoas abscesses. While the primary psoas abscess is defined as an abscess of unknown origin, its diagnostic symptoms and signs are almost identical to those of the secondary psoas abscess^[9,10]. For example, the insidious onset of abscess formation is not responsive to medical treatment, the classical triads of psoas abscess such as fever, flank pain, and limitation of hip movement, can all present in both primary and secondary psoas abscesses^[6-8,11]. After reviewing the information from the literature, we found that only the results of bacterial culture, if could be obtained before surgery, could indicate the nature of the abscess being primary or secondary. The most common causative pathogen of primary psoas abscess is *Staphylococcus aureus*, while that of secondary psoas abscess is usually mixed intestinal floras^[9,10,12,18].

Second, as CT scan is used as a modality in addition to the physical signs for definite diagnosis in most of the reported cases, there is no doubt that CT scan of the abdomen with contrast is the most widely-used imaging study with the highest accuracy and efficiency^[5-10,19]. CT scan of the abdomen not only helps in the establishment of the diagnosis, but also in the evaluation of the extension of involvement and in its treatment. For example, application of advanced high speed helical CT (which was the case in this report and has never been demonstrated before) can demonstrate a reconstructed coronal image that is of great help for surgical planning because it simulates the operation field. In addition, the drainage of abscess can be achieved by percutaneous and retroperitoneal approach or by laparotomy based on CT findings.

Third, whether the abscess is managed surgically or non-surgically should be carefully evaluated. With the improving technique and the accumulating experiences of interventional radiology, there are several reports demonstrating substantial results by percutaneous drainage of the abscess and then by surgery only if percutaneous drainage fails or is contraindicated. For example, Benoist *et al*^[20] demonstrated that percutaneous drainage can drain 81% postoperative abdominal abscesses in patients without sepsis at presentation. Cantasdemir *et al*^[21] showed that primary and secondary iliopsoas abscesses can be successfully treated with percutaneous drainage in patients without secondary abdominal pathology. Percutaneous drainage of the abscesses is less invasive. Gerzof *et al*^[22] have recommended that percutaneous drainage should be done for most complex abscesses, but we are still

uncertain if the percutaneous approach is adequate for our patient, because our patient was in a critical condition requiring prompt drainage of the abscess which cannot be achieved by percutaneous method and the infection could not be controlled if there was a persistent existing focus as previously reported by Ushiyama *et al*^[15]. In addition, according to the results reported by Benoist *et al*^[20] and Cantasdemir *et al*^[21], a combined therapy (that is drainage in combination with surgery) might be contraindicated in our patient because he was septic and had a persistent existing intra-abdominal focus. Hence, it is our experience that surgery is advantageous over percutaneous drainage in patients under critical conditions such as perforated appendicitis, diverticulitis or malignancy^[14].

Fourth, though abscesses inside the thigh is due to direct extension from retroperitoneum, it might be wise to create a separate incision at the thigh to drain the abscess rather than from the trunk. Draining thigh abscess by an incision at the thigh has two advantages. First, the abscess can be more easily and directly approached. Second, the viability of muscle and fascia of the thigh as well as the need for further debridement can be adequately evaluated. This is supported by the fact that some of thigh abscesses can be cured by drainage alone^[6,7], while others with extensive myonecrosis require debridement or amputation^[5,13].

In conclusion, formation of huge retroperitoneal abscesses with thigh involvement is a serious complication of perforated acute appendicitis. To improve the treatment outcome, patients with retroperitoneal infection should undergo CT scan in order to find the origin of the infection and to choose the best way to drain the abscess.

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CASE REPORT

Management of patients with stercoral perforation of the sigmoid colon: Report of five cases

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Abstract

To our knowledge, stercoral perforation of the colon is rarely seen with fewer than 90 cases reported in the literature till date. We explored the principles of management to prevent impending mortality in five patients with this condition. Five patients, two males and three females, whose median age was 64 years, had sustained stercoral perforation of the sigmoid colon. Chronic constipation was the common symptom among these patients. Three patients underwent a Hartmann's procedure and another two were treated with segmental colectomy with anastomosis and diverting colostomy. There was one surgical mortality and the other patients had an uneventful hospital stay. Timely intervention to prevent and/or treat any associated sepsis along with extensive peritoneal lavage and surgical intervention to remove diseased colonic tissue at the primary stercoral ulceration site coupled with aggressive therapy for peritonitis are key treatment modalities in salvaging patients presenting with stercoral perforation of the colon.

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Key words: Stercoral perforation; Colon; Management

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INTRODUCTION

Berry^[1] presented the first case of stercoral perforation of the colon to the Pathological Society of London in 1894, and it is a rare event with fewer than 90 cases reported in the literature through 2002^[2]. Stercoral perforation of the colon is an aggravating condition following successive bowel wall ischemic necrosis by fecal mass^[3], and dictates a rather poor clinical outcome in patients with a compromised general condition^[4,5]. We reviewed the history of five patients of stercoral perforation of colon at our hospital, in order to elucidate the appropriate intraoperative procedures and obtain clinical results which will enhance the percentage of accurate preoperative diagnosis and clarify the most feasible perioperative management.

CASE REPORTS

During the years 2001-2005, 22 patients with free perforation of the colon were hospitalized at Chang Gung Memorial Hospital in Chiayi. The etiology of perforation was cancer in nine patients, ruptured diverticulitis in five, iatrogenic colon perforation related to colonoscopy procedure in two, one patient with traumatic colon perforation, and the other five patients were diagnosed as stercoral perforation which fit the diagnostic criterion proposed by Maurer *et al*^[3].

These five patients formed the basis for this brief report (Tables 1 and 2). There were two men and three women aged between 4-84 years. All gave a long history of serious and chronic constipation except for a little girl. Four patients also had concomitant diseases that might have enhanced the development of chronic constipation such as diabetic enteropathy, hypothyroidism, and hemiparesis sequelae of stroke.

These five patients presented with sudden and severe abdominal pain. On physical examination, diffuse peritonitis was present in all the patients along with high fever and leukocytosis. Four patients were found to have sub-diaphragmatic free air on a standing chest roentgenogram, but only one showed localized extraluminal air on abdominal CT scanning. All patients underwent emergency laparotomy by a general surgeon with a preoperative diagnosis of hollow organ perforation. The colorectal surgeon was summoned during surgery to verify an intraoperative finding of colonic perforation. All perforation sites were found to be located at the

Table 1 Perioperative data on patients with stercoral perforation of the colon

| Patient Number | Gender | Age | Interval (day) ¹ | Case history | X-ray finding | Peritonitis | Localization | Perforation size (cm) and site | Fecaloma |
|----------------|--------|-----|-----------------------------|---|---------------------------|---------------|-----------------------------|--------------------------------|--------------------------------|
| A | F | 4 | 1 | Chronic constipation | Subdiaphragmatic free air | Generalized | Mid-sigmoid Colon | 1×0.8 Anti-mesocolic | Within abdominal cavity |
| B | M | 70 | 3 | Chronic constipation, Cushing's syndrome | Subdiaphragmatic free air | Generalized | Rectosigmoid colon junction | 5×3 Anti-mesocolic | Within the colon |
| C | F | 84 | 2 | Chronic constipation, D.M. | Subdiaphragmatic free air | Generalized | Sigmoid Colon | 3×2, Anti-mesocolic | Protruding through perforation |
| D | F | 79 | 1 | Chronic constipation, stroke | Subdiaphragmatic free air | Lower abdomen | Mid-sigmoid Colon | 2.5×1.5, Anti-mesocolic | Within the colon |
| E | M | 64 | 2 | Chronic constipation, D.M., hypothyroidism, gouty arthritis | Extraluminal free air | Generalized | Sigmoid Colon | 4×2, Anti-mesocolic | Within the colon |

¹ between symptom to operation.

Table 2 Perioperative data of patients with stercoral perforation of the colon

| Patient number | Pathology | Ascites culture ¹ | Colonoscopy ² | Stercoral ulcer at proximal colon | Operation procedures | Peritoneal lavage ³ | Complication |
|----------------|-------------------|---|----------------------------|---|---|--------------------------------|---|
| A | Fecal peritonitis | <i>E. coli</i> , <i>Enterococcus faecalis</i> , <i>B. Fragilis</i> | No | Undetectable | Segmental colectomy+diverting enterostomy | Plenty | Nil |
| B | Purulent ascites | <i>Enterococcus faecalis</i> , <i>B. fragilis</i> | Yes (65, A-colon) | Four shallow stercoral ulceration diffusely | Hartmann's operation+rectal mucus fistula | Moderate | Mortality (overwhelming sepsis at post-op 21st d) |
| C | Fecal peritonitis | <i>E. coli</i> | Yes (50, proximal T-colon) | No ulceration | Hartmann's operation+rectal mucus fistula | Massive | Superficial wound infection |
| D | Purulent ascites | <i>E. coli</i> , <i>Kleb. pneumoniae</i> , <i>B. thetaiotaomicron</i> | Yes (75, A-colon) | No ulceration | Segmental colectomy+diverting enterostomy | Massive | Superficial wound infection |
| E | Purulent ascites | <i>E. coli</i> , <i>Enterococcus faecium</i> , <i>Bacteroides sp.</i> | Yes (80, A-colon) | No ulceration | Hartmann's operation+rectal mucus fistula | Massive | Fascial dehiscence |

¹Heavy flora cultured as. ²(cm) distance at the most proximal to perforation and location. ³Massive: >10 000 mL; moderate: <6 000 mL; plenty: 1 500 mL.

antimesenteric part of the sigmoid colon with perforations ranging in size from 2 to 5 cm. The perforation margins were well circumscribed with no local inflammation or any other chronic process. Three patients underwent a Hartmann's operative procedure with colostomy and rectal mucous fistula, another two patients were treated with segmental resection of the diseased colon with anastomosis and diverting enterostomy.

During the operation four patients, with the exception of the little girl, underwent colonoscopy to clarify the extent of the lesion of severe stercoral ulceration with impending colon perforation. Colon lavage was also performed as permitted by the proximity of the lesion.

Hemodynamic improvement and reversal of hypotensive status was noted immediately after colectomy was performed.

Intraperitoneal irrigation with massive amounts of warm normal saline solution and adequate placement of drainage tubes preceded the closure of the celiotomy incision.

All patients received broad-spectrum intravenous antibiotics and nutritional support for the duration of their hospital stay. Diverting enterostomy was closed in two patients 3 mo later. Hartmann's procedure was reversed in two patients after a minimum time of one and a half years. There were one surgical mortality due to overwhelming sepsis, morbidities due to superficial wound infection in two patients and fascial dehiscence in one patient, respectively.

DISCUSSION

Colon perforation is a rather uncommon event usually caused by malignancy, amoebic colitis, diverticular disease, spontaneous perforation, stercoral ulceration, steroid therapy, trauma, and ulcerative colitis^[4]. The usual definition of stercoral perforation is "Perforation of the large bowel due to pressure necrosis from a fecal mass"^[6], and represents a cause of colon perforation. Stercoral perforation of the colon was very rare for it was

first described in 1894 and less than 90 cases have been reported in the literature till date. We had five patients (22%) with this condition presenting as colonic perforation (22 patients) at our hospital, and it was said to be the largest series involving stercoral perforation of the colon in a single medical institute within a short-term interval.

Severe chronic constipation is considered to be the main causative factor in the development of stercoral perforation of the colon^[4,6-8]. In our series, each patient had a long history of chronic constipation requiring medication for relief; the youngest female patient did not have congenital Hirschsprung's disease based on the pathologic examination of the colectomy specimen.

Long-standing constipation may enhance the formation of stone-hard fecalomas and maintain a persistent pressure over the bowel wall leading to pressure necrosis of the mucosa. Nevertheless, stercoral ulceration of colonic mucosa does not always occur among constipation cases, and not every stercoral ulceration results in colon perforation.

Multiple fecalomas can result in multiple stercoral ulcerations^[6]. One of our five patients and 28% of the cases found in the literature showed this characteristic feature. There are several reasons why the perforation sites are located in the antimesenteric aspect and in the sigmoid colon: (1) Hypoperfusive status existing in the antimesenteric aspect other than the mesenteric border; (2) due to its more distal location and more solid consistency, fecalomas tend to form in more distal aspects of the large intestine such as the sigmoid colon; (3) being the most narrow region of the entire large intestine stool has difficulty passing through the sigmoid colon which increases the intraluminal pressure to the point where it can compress submucosal capillary vessels and reduce perfusion of the colonic wall; (4) prolonged localized pressure on the colon wall causing pressure ulcerations to appear^[3,5,9]. In this series of patients perforation was only found in the anti-mesenteric margin of the sigmoid colon and not in other colonic site.

Treatment of intra-abdominal sepsis was achieved by massive saline irrigation and perforation control with the intention of decreasing the bacterial load in the abdominal cavity and deterring the development of overwhelming sepsis^[10].

The single mortality in this series differed from the other four cases in the reduced amount of peritoneal lavage received due to intraoperative hypothermia.

Broad spectrum antibiotics were initiated in all the patients after hospital admission for polymicrobial peritoneal cavity contamination resulting from intestinal perforation^[11].

In reviewing the literature, it was noted that intraoperative colonoscopy to inspect the remainder of the colon for stercoral ulceration was not undertaken in cases of stercoral perforation of the colon. To our knowledge, we are the first group to perform intraoperative colonoscopic examination.

The purpose of intraoperative colonoscopic examination was to ensure the adequacy of the colonic resection and rule out the presence of additional stercoral ulcerations that might lead to delayed colonic perforation.

Intraoperative colonoscopy was performed within 10 min after the colon was cleared of impacted stools.

Only one patient was found to have more than one stercoral ulceration of the entire colon. Colectomy was performed to remove all stercoral ulcerated lesions in this patient, unfortunately, due to an immuno-compromised state the patient expired after developing overwhelming sepsis.

Maurer *et al*^[3] postulated that colonic dilation and the presence of multiple fecalomas may indicate such additional stercoral ulceration and carried the risk of a second perforation. We concur with this point of view based on the findings during intraoperative colonoscopy in our series and recommend removing pathologically altered or dilated colon segments to prevent another episode of colon stercoral perforation.

Mortality related to colon stercoral perforation was reported to be high^[4,6,9]. Analysis of the reports in the literature revealed additionally that 28% of patients with stercoral perforation of the colon have multiple stercoral ulcers in the colon and that substantial mortality is encountered if only minor surgical procedures are employed as treatment such as simple exteriorization of the perforation site or closure of perforation hole with the addition of a diverting enterostomy^[6,9].

Two review articles^[6,9] and one series^[3] with optimal clinical outcome in relation to the treatment of colonic stercoral perforation recommended that resection of the colon segment with an end colostomy and either mucous fistula or Hartmann's closure of the rectum would encounter comparatively low mortality. In our series, we took surgery of resection of colon with anastomosis and loop diverting colostomy in two patients (case 1 and case 4) with limited intraperitoneal septic condition and acceptable general condition. There were some advantages doing this procedure such as simple closure of colostomy someday. The other three patients were performed Hartmann's procedure with rectal mucous fistula.

In our series, we undertook surgical colonic resection with anastomosis and loop diverting colostomy in two patients (case 1 and case 4) with limited intraperitoneal sepsis and an acceptable general medical condition. The advantage of performing this procedure resides in the possibility of performing a simple closure of the colostomy in the future. The other three patients underwent Hartmann's procedure with rectal mucous fistula.

Stercoral perforation of the colon is not purely a surgical condition as it is usually complicated by a medical illness such as a superimposed immuno-compromised state which imparts a poor prognosis. Despite efforts to correct Cushing's syndrome, sufficient nutritional support and aggressive antibiotics therapy, it was not possible to prevent the systemic sepsis in case 2.

In conclusion, a favorable outcome in the treatment of stercoral perforation depends upon: (1) immediate treatment of any underlying sepsis; (2) removal of all stercoral ulcerated diseased colonic tissue; (3) extensive peritoneal lavage; (4) aggressive therapy to counteract colonic perforation peritonitis and (5) appropriate treatment of any co-morbid medical conditions. Prompt

institution of these measures will enhance survival of the patient and contribute to achieving a low mortality rate in stercoral perforation of the colon.

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- 3 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaobua Zazhi* 1999; 7: 285-287

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Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

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| Number | Nonstandard | Standard | Notice |
|--------|--|--|--|
| 1 | 4 days | 4 d | In figures, tables and numerical narration |
| 2 | 4 days | four days | In text narration |
| 3 | day | d | After Arabic numerals |
| 4 | Four d | Four days | At the beginning of a sentence |
| 5 | 2 hours | 2 h | After Arabic numerals |
| 6 | 2 hs | 2 h | After Arabic numerals |
| 7 | hr, hrs, | h | After Arabic numerals |
| 8 | 10 seconds | 10 s | After Arabic numerals |
| 9 | 10 year | 10 years | In text narration |
| 10 | Ten yr | Ten years | At the beginning of a sentence |
| 11 | 0,1,2 years | 0,1,2 yr | In figures and tables |
| 12 | 0,1,2 year | 0,1,2 yr | In figures and tables |
| 13 | 4 weeks | 4 wk | |
| 14 | Four wk | Four weeks | At the beginning of a sentence |
| 15 | 2 months | 2 mo | In figures and tables |
| 16 | Two mo | Two months | At the beginning of a sentence |
| 17 | 10 minutes | 10 min | |
| 18 | Ten min | Ten minutes | At the beginning of a sentence |
| 19 | 50% (V/V) | 500 mL/L | |
| 20 | 50% (m/V) | 500 g/L | |
| 21 | 1 M | 1 mol/L | |
| 22 | 10 μM | 10 μmol/L | |
| 23 | 1N HCl | 1 mol/L HCl | |
| 24 | 1N H ₂ SO ₄ | 0.5 mol/L H ₂ SO ₄ | |
| 25 | 4rd edition | 4 th edition | |
| 26 | 15 year experience | 15- year experience | |
| 27 | 18.5 kDa | 18.5 ku, 18 500u or M:18 500 | |
| 28 | 25 g.kg ⁻¹ /d ⁻¹ | 25 g/(kg·d) or 25 g/kg per day | |
| 29 | 6900 | 6 900 | |
| 30 | 1000 rpm | 1 000 r/min | |
| 31 | sec | s | After Arabic numerals |
| 32 | 1 pg L ⁻¹ | 1 pg/L | |
| 33 | 10 kilograms | 10 kg | |
| 34 | 13 000 rpm | 13 000 g | High speed; g should be in italic and suitable conversion. |
| 35 | 1000 g | 1 000 r/min | Low speed. g cannot be used. |
| 36 | Gene bank | GenBank | International classified genetic materials collection bank |
| 37 | Ten L | Ten liters | At the beginning of a sentence |
| 38 | Ten mL | Ten milliliters | At the beginning of a sentence |
| 39 | umol | μmol | |
| 40 | 30 sec | 30 s | |
| 41 | 1 g/dl | 10 g/L | 10-fold conversion |
| 42 | OD ₂₆₀ | A ₂₆₀ | "OD" has been abandoned. |
| 43 | One g/L | One microgram per liter | At the beginning of a sentence |
| 44 | A260 nm ^b P<0.05 | A ₂₆₀ nm ^a P<0.05 | A should be in italic. In Table, no note is needed if there is no significance instatistics: ^a P<0.05, ^b P<0.01 (no note if P>0.05). If ther is a second set of P value in the same table, ^c P<0.05 and ^d P<0.01 are used for a third set: ^a P<0.05, ⁱ P<0.01. |
| 45 | [*] F=9.87, [§] F=25.9, [#] F=67.4 | ¹ F=9.87, ² F=25.9, ³ F=67.4 | Notices in or under a table |
| 46 | KM | km | kilometer |
| 47 | CM | cm | centimeter |
| 48 | MM | mm | millimeter |
| 49 | Kg, KG | kg | kilogram |
| 50 | Gm, gr | g | gram |
| 51 | nt | N | newton |
| 52 | l | L | liter |
| 53 | db | dB | decibel |
| 54 | rpm | r/min | rotation per minute |
| 55 | bq | Bq | becquerel, a unit symbol |
| 56 | amp | A | ampere |
| 57 | coul | C | coulomb |
| 58 | HZ | Hz | |
| 59 | w | W | watt |
| 60 | KPa | kPa | kilo-pascal |
| 61 | p | Pa | pascal |
| 62 | ev | EV | volt (electronic unit) |
| 63 | Jonle | J | joule |
| 64 | J/mmol | kJ/mol | kilojoule per mole |
| 65 | 10×10×10cm ³ | 10 cm×10 cm×10 cm | |
| 66 | N·km | KN·m | moment |
| 67 | x±s | mean±SD | In figures, tables or text narration |
| 68 | Mean±SEM | mean±SE | In figures, tables or text narration |
| 69 | im | im | intramuscular injection |
| 70 | iv | iv | intravenous injection |
| 71 | Wang et al | Wang <i>et al.</i> | |
| 72 | EcoRI | EcoRI | <i>Eco</i> in italic and RI in positive. Restriction endonuclease has its prescript form of writing. |
| 73 | Ecoli | <i>E.coli</i> | Bacteria and other biologic terms have their specific expression. |
| 74 | Hp | <i>H pylori</i> | |
| 75 | Iga | Iga | writing form of genes |
| 76 | igA | IgA | writing form of proteins |
| 77 | ~70 kDa | ~70 ku | |

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Molecular therapy for hepatic injury and fibrosis: Where are we?

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Abstract

Hepatic fibrosis is a wound healing response, involving pathways of inflammation and fibrogenesis. In response to various insults, such as alcohol, ischemia, viral agents, and medications or hepatotoxins, hepatocyte damage will cause the release of cytokines and other soluble factors by Kupffer cells and other cell types in the liver. These factors lead to activation of hepatic stellate cells, which synthesize large amounts of extracellular matrix components. With chronic injury and fibrosis, liver architecture and metabolism are disrupted, eventually manifesting as cirrhosis and its complications. In addition to eliminating etiology, such as antiviral therapy and pharmacological intervention, it is encouraging that novel strategies are being developed to directly address hepatic injury and fibrosis at the subcellular and molecular levels. With improvement in understanding these mechanisms and pathways, key steps in injury, signaling, activation, and gene expression are being targeted by molecular modalities and other molecular or gene therapy approaches. This article intends to provide an update in terms of the current status of molecular therapy for hepatic injury and fibrosis and how far we are from clinical utilization of these new therapeutic modalities.

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Key words: Fibrosis; Gene therapy; Hepatic stellate cell; Hepatocyte; Injury

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INTRODUCTION

Hepatic fibrosis is a wound-healing process after chronic liver injury, and is characterized by the activation of hepatic stellate cells (HSC) and excess production of extracellular matrix (ECM) components. The activation of HSC involves the transdifferentiation from a quiescent state into myofibroblast-like cells with the appearance of smooth muscle α -actin (SMA) and loss of cellular vitamin A storage^[1]. The activated HSC are distinguished by accelerated proliferation and enhanced production of ECM components. Cross-talks between parenchymal and non-parenchymal cells constitute the major interactions in the development of hepatic injury and fibrosis. Soluble factors, such as cytokines, chemokines or reactive oxygen species (ROS) are the intermediates in these cross-talks, and are possible targets for therapeutic consideration. A schematic illustration shown in Figure 1 represents the major cascades of the injury process, including the interactions between damaged hepatocytes and Kupffer cell activation, proliferation and activation of HSC, and the excess production of ECM components during hepatic fibrosis. Along with these cascades, possible molecular therapeutic targets are provided in *italics*. From our improved understanding of the mechanisms underlying liver injury and fibrosis, key steps, such as signaling, activation, and gene expression in specific cell types of the liver, have been targeted by molecular modalities^[2,3]. However, various molecular therapeutic approaches carry advantages and disadvantages and patient safety is the primary concern in considering the use of novel modalities^[4]. Thus, people may ask: "What is the current status of molecular therapy for hepatic injury and fibrosis?" "How far from now these novel modalities will be possibly utilized in patients?" This concise review intends to answer these questions.

TARGETING SPECIFIC MOLECULES OR PATHWAYS

Inhibition of hepatocellular apoptosis

Chronic hepatocellular death via necrosis and/or apoptosis initiates inflammatory responses and hepatic fibrogenesis. Ideally, hepatocytes regenerate and replace dying cells; however, hepatocellular regeneration in chronic liver injury is often inhibited due to the imbalance of growth factors and distortion of liver architecture and circulation. Cellular debris and apoptotic bodies accumulate and initiate inflammatory responses, which may form a self-amplifying loop that further compromises recovery from injury, and facilitates the fibrogenic process^[5]. HSC may

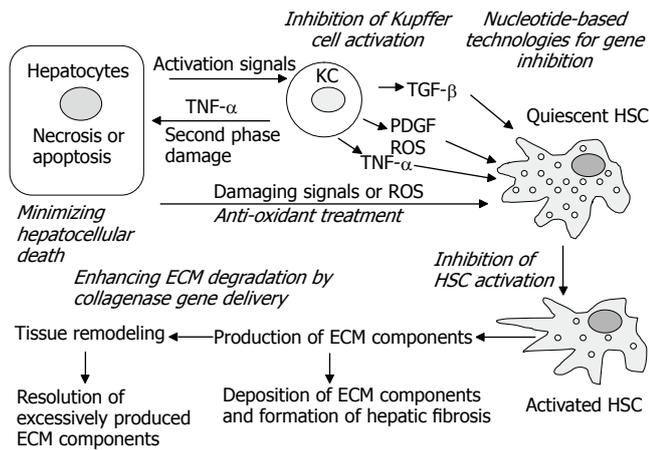


Figure 1 Strategies for the prevention and treatment of hepatic fibrosis. A simplified version of the pathogenesis of hepatic fibrosis is illustrated schematically. At various stages, possible therapeutic strategies are provided in *italics*. See the text for details. KC = Kupffer cells; ECM = extracellular matrix; HSC = hepatic stellate cells; PDGF = platelet-derived growth factor; ROS = reactive oxygen species; TNF- α = tumor necrosis factor- α ; TGF- β = transforming growth factor- β .

engulf the apoptotic bodies, which has been demonstrated *in vitro*, and phagocytosis of apoptotic bodies by quiescent HSC facilitates the phenotypic transdifferentiation to myofibroblasts^[6]. Activated HSC with engulfed apoptotic bodies were identified in a rat model of bile-duct ligation and liver biopsy specimens from HCV infection^[7].

Tumor necrosis factor- α (TNF- α) (via TNF-receptor-1) can initiate apoptosis^[8,9]. This extrinsic pathway is signaled through cell surface death receptors^[9], including Fas (also known as CD95), TNF- α -receptor-1, and TNF- α -related-apoptosis-inducing-ligand receptors 1 & 2 (TRAIL-R1 and R2). Examples of this extrinsic pathway of programmed cell death include autoimmune hepatitis, viral hepatitis^[10,11], chronic alcohol consumption, D-galactosamine (GalN) plus lipopolysaccharide (LPS)-induced acute liver injury, and ischemia/reperfusion-associated liver injury. In these forms of injury, cytotoxic cytokines or chemokines play a crucial role in the mediation of the injury process^[12-18]. In contrast to the extrinsic apoptotic pathway, the intrinsic pathway is based on damage or dysfunction of intracellular organelles, such as lysosomes, endoplasmic reticulum, nucleus and mitochondria^[19]. This mechanism involves changes in membrane permeability and integrity of the subcellular organelles. Examples are that damage to nuclear or mitochondrial DNA can initiate apoptosis, and that the release of cytochrome C from mitochondria triggers apoptotic cascades. This intrinsic pathway of programmed cell death is often seen in drug toxicity or hepatotoxin-induced liver injury, such as acetaminophen overdose, alcohol toxicity, *etc*^[18,20].

Inhibiting apoptosis will alleviate liver injury, and in turn, delay or stop the progression of hepatic fibrosis. Molecular therapy has specifically targeted various steps in the apoptosis pathway including inhibition of cell death receptors, mediators of the apoptosis cascade, and regulation of the apoptosis cascade. One target is to inhibit Fas receptor expression, which signals the initiation of the apoptosis cascade. Another possible target includes inhibition of caspase expression. Caspases are proteolytic

enzymes classified according to their function into ICE-like caspases (caspase 1, 4, 5, 13), death signaling or initiator caspases (caspase 2, 8, 9, 10), and death effector or executioner caspases (caspase 3, 6, 7).

Zhang *et al*^[21] employed antisense oligodeoxynucleotide (ODN) ISIS 22023, specific for mouse Fas to prevent Fas ligand (Jo2 or CD95)-induced fulminant liver failure and mortality. Antisense ODN treatment reduced Fas mRNA and protein expression in the liver by 90%, and completely prevented mortality which was 100% in the control group with extensive hepatocyte apoptosis and liver hemorrhage. In a separate study, Song *et al*^[22] evaluated small interfering RNA (siRNA) against Fas for its ability to inhibit apoptosis and protect the liver in the same animal model. siRNA against the Fas receptor was administered via tail vein injection, which led to 88% of uptake by hepatocytes. The injection decreased Fas mRNA expression by 8 to 10-fold when compared to controls, and the decline in Fas mRNA and Fas protein was sustained for up to 10 d. Control mice displayed bridging fibrosis, whereas mice receiving anti-Fas siRNA injections had no evidence of necrosis or fibrosis. Mice treated with Fas-specific siRNA also had an improved mortality. Eighty-two percent of mice survived over 10 d of observation, whereas all control mice died within 3 d of lethal challenge with Jo2. Thus, both studies demonstrate that the inhibition of Fas receptor synthesis by either specific antisense ODN or siRNA protects animals from Jo2-induced fulminant liver failure and mortality^[21-22].

Regulators of apoptosis in the Bcl-2 family have also been targeted with molecular approaches. Bcl-2 is a member of the proto-oncogene family, which blocks cytochrome C release during apoptosis signaling. Several gene products in the Bcl-2 family inhibit apoptosis, including Bcl-xL, Bcl-w, A1, Mcl-1 and Boo, whereas others, such as Bax, Bak, Bok, Bik, Bad and Bid, promote apoptosis^[23]. Zhang *et al*^[24] evaluated effects of the antisense against Bcl-xL (ISIS16009) and Bid on Jo2-induced fulminant liver failure in mice. Pre-administration of ISIS16009 reduced liver Bcl-xL expression, potentiated liver apoptosis and increased lethality in this mouse model of Fas-mediated fulminant hepatitis. Antisense ODN against Bid caused an 80% decrease in Bid expression. The reduction of Bid expression by the antisense treatment protected mice from Fas-mediated fulminant hepatitis and death with 100% survival in treated mice^[24]. Therefore, Bid seems to be a reasonable target for reducing liver injury in which apoptosis is a predominant pathway of cell death, such as Fas ligand-induced acute liver injury.

Caspase 8 is a downstream target of death receptors and is important in the mediation of apoptosis by Fas and TNF- α . Zender *et al*^[25] evaluated the effects of siRNA against caspase 8 in acute liver damage induced by Jo2 and adenovirus expressing Fas ligand (AdFasL). siRNA against specific caspase 8 target sequences was transfected into Hep G2 cells, and the transfected cells were further infected with AdFasL. Their results confirmed that the inhibition of caspase 8 by siRNA prevented Fas-mediated apoptosis in Hep G2 cells. The animal experiments involved siRNA delivery and administration of Fas antibody or AdFasL. The siRNA delivery resulted in a greater than 3-fold reduction in caspase 8 mRNA

expression. Improved survival was significant even when caspase 8 siRNA was administered during ongoing acute liver failure. In addition, the study is of particular interest in that caspase 8 siRNA treatment was successful not only in acute liver failure mediated by specific Fas agonistic agents (Jo2 and AdFasL), but also in acute liver failure caused by an injection of a large amount of wild-type adenoviruses.

Transforming growth factor- β (TGF- β)

TGF- β is the most potent cytokine for enhancing hepatic fibrogenesis. It suppresses hepatocyte proliferation, stimulates the activation of HSC, promotes ECM production, and mediates hepatocyte apoptosis^[26]. Smad3 (a transcriptional factor in TGF- β receptor downstream signal transduction) knock-out mice did not develop hepatic fibrosis^[27]; while, transgenic mice overexpressing TGF- β 1 developed hepatic fibrosis faster than wild type mice, and the fibrosis regressed slower after the withdrawal of the fibrogenic agent^[28-30]. Many different strategies of molecular therapy have focused on the inhibition of TGF- β effects by blocking its synthesis, using TGF- β binding proteins, soluble receptors, or targeting its downstream signal transduction pathways.

Qi *et al.*^[31] evaluated adenoviral expression of truncated TGF- β receptor type II to abolish TGF- β signaling in liver fibrosis mediated by dimethylnitrosamine (DMN). DMN induces fatty degeneration of hepatocytes, activation of HSC, macrophage infiltration and secretion of TGF- β . Sprague-Dawley rats were given a single infusion of adenoviral vectors (AdCAT β -TR) encoding the TGF- β receptor II gene (TGF- β RII) via portal vein injection. A greater than 20-fold increase in truncated receptor mRNA expression with the adenoviral gene delivery was detected in animals after the TGF- β receptor type II gene delivery with adenoviral vectors. The gene delivery improved the animal mortality, decreased liver hydroxyproline content by 3.4-fold, hyaluronate levels by nearly 20-fold, AST by 100-fold, and ALT by 63-fold. Less hepatocyte injury and fibrosis with a decreased infiltration of monocytes/macrophages and reduced semiquantitative score of fibrosis in histopathology were reported, which was consistent with decreased gene expression of collagen type I, fibronectin, smooth muscle α -actin (SMA), and TGF- β 1 in the treated animals. Yata *et al.*^[32] administered a soluble TGF- β type II receptor (STR) protein in CCl₄-induced liver fibrogenesis in BALB/c mice. Elevated STR levels were associated with a reduction in procollagen type I mRNA expression in the mouse liver. In the 8-wk study, lower concentrations of STR had the greatest effect on procollagen type I mRNA expression. Quantitative morphometrics showed that lower concentrations of STR were the most anti-fibrogenic. These findings verified the antifibrotic effect of inhibiting TGF- β in chronic hepatic injury.

Tumor necrosis factor- α (TNF- α)

TNF- α is a key cytokine involved in many forms of liver injury, and may play a crucial role in HSC activation and hepatocyte regeneration^[33-34]. The major cell type for TNF- α production in the liver is Kupffer cells which release TNF- α when activated by factors released by

damaged hepatocytes, and by ROS occurrence. TNF- α participates in the second phase of hepatocellular damage via apoptosis and TRAIL receptor activation in alcoholic or other hepatotoxin-induced liver injury^[35]. It acts on HSC, and may contribute to the activation process^[36]. Accordingly, reducing TNF- α production, or blocking its action will significantly minimize liver injury caused by alcohol toxicity, acetaminophen overdose, or ischemia/reperfusion-associated liver injury in animal models^[35, 37].

Ponnappa *et al.*^[38] has developed a pH-sensitive liposomal formulation consisting of phosphatidyl ethanolamine, cholesteryl hemisuccinate, and cholesterol in a specific ratio (7:4:2), to deliver antisense ODN against TNF- α . This liposomal formulation, which is characterized by its feature of lysosomal resistance, helps release encapsulated-antisense ODN (TJU-2755) into the cytoplasm of Kupffer cells. Rats that received pretreatment with liposome-encapsulated TJU-2755 had a decreased expression of TNF- α mRNA and TNF- α secretion in response to LPS challenge. In subsequent studies, the *in vivo* efficacy of TJU-2755 was assessed against LPS-induced liver damage in ethanol-fed rats^[39]. Liver damage was induced in male Lewis rats fed an ethanol-containing liquid diet for 8-10 wk followed by an intravenous injection of LPS. Pretreatment of the animals with TJU-2755 encapsulated in pH-sensitive anionic liposomes prevented liver damage by 60-70%, as assessed by the release of liver enzymes and histology. These results indicate that pH-sensitive, anionic liposomes can be used to effectively deliver TNF- α antisense ODN against TNF- α -mediated liver injury^[39].

Platelet-derived growth factor (PDGF)

PDGF is the most potent mitogen for HSC with effects in growth stimulation, chemotaxis, and intracellular signaling. PDGF expression is upregulated in hepatic injury, as are PDGF receptors in activated HSC. The tyrosine kinase activity of PDGF receptors signals through PI-3K, Ras, Raf-1, and ERK, leading to nuclear translocation and activation of nuclear transcription factors^[40]. Interrupting PDGF signal effects with specific agents inhibiting tyrosine phosphorylation, or interrupting down-stream signal transduction pathways attenuates hepatic fibrosis by preventing proliferation and chemotaxis of HSC *in vitro* and *in vivo*^[40]. Selectively targeting the PDGF receptor by specific antibodies or agents has been considered to be a valuable strategy to block the progression of hepatic fibrogenesis^[41-42].

Borkham-Kamphorst *et al.*^[43] cloned a chimera of PDGF receptor type β gene, fused to the IgG-Fc domain of human immunoglobulin, into an adenoviral vector. The gene product, soluble PDGF receptor, has a high ligand binding capacity, 100 to 1000-fold higher than that of extracellular monomeric PDGF receptors. The effects of soluble PDGF receptor gene therapy on PDGF-induced DNA synthesis, tyrosine autophosphorylation, and activation of ERK, P13K, and protein kinase B in HSC were evaluated *in vitro*. The *in vitro* studies with rat HSC transfected with AdSPDGFR showed decreased levels of PDGF-BB mRNA and thrombospondin-1 with lowered expression of collagen type I mRNA. The receptor gene

delivery, however, did not affect levels of SMA or desmin levels in HSC, indicating that it did not significantly affect the HSC activation^[43]. In their subsequent study, antisense against PDGF β -chain incorporated into adenoviral vector serotype 5 (Ad5-CMV-PDGF) was transduced in cultured rat HSC^[44]. The antisense clearly exhibited the ability to down-regulate endogenous PDGF β -chain and PDGF R- β mRNA as well as procollagen type I gene expression in culture-activated HSC and rat livers^[44].

Kupffer cells

Due to the critical role of Kupffer cells in the mediation of liver injury and fibrogenesis, inhibiting Kupffer cell activity has been proposed as a means to reduce liver injury. Ogushi *et al*^[45] modulated Kupffer cell function by administering double stranded antisense ODN targeting NF- κ B, the p65 subunit. The mice were first sensitized with intraperitoneal injection of heat killed *Propionibacterium acnes*. Phosphorothioate modified antisense ODN was delivered with hemagglutinating virus of Japan (HVJ)-liposomal complexes via portal vein injection on d 4 and 7 after *P acnes* priming challenge. The treated mice also had decreased levels of activated NF- κ B translocation as shown by p65 staining. Then, the mice were injected with LPS and sacrificed later. The HVJ-liposome-mediated antisense ODN against NF- κ B significantly improved animal survival, and the mortality was decreased to 15% in mice treated with NF- κ B decoy ODN as compared to 90% in control mice that died within 24 hours. The treatment with decoy antisense ODN against NF- κ B also suppressed the production of proinflammatory cytokines (IL-1 β , TNF- α , IL-18, and IL-12) by Kupffer cells and decreased mRNA expression of IFN- γ and Fas-L. Less infiltration of inflammatory cells and destruction of sinusoidal architecture were documented by histopathology in the animals treated with antisense ODN against NF- κ B^[45].

REACTIVE OXYGEN SPECIES AND FREE RADICAL SCAVENGERS

Oxidative stress is an important mechanism in liver injury. ROS include superoxide anions, hydroxyl radicals, hydrogen peroxide, and hydroxyethyl radicals (HER), and are generated from a variety of insults such as drug/toxin metabolites, ischemia/reperfusion, and alcohol metabolism. ROS are involved in necrosis and apoptosis of hepatocytes, and contribute to HSC activation^[2,46]. Several major classes of free radical scavengers, such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GSH-P), as well as SOD mimics, were investigated in various forms of liver injury, and they afforded effective protection against the oxidative insults to the tissue^[47-48].

Three isoforms of SOD exist. Copper/Zinc (Cu/Zn) SOD is localized to the cytosol and nucleus, manganese (Mn) SOD is localized to the mitochondria and, extracellular (EC) SOD is primarily localized to the interstitial matrix. We employed polycationic liposomes previously formulated by our group with polycationic lipids (PCL)^[49] to deliver the EC-SOD gene for the prevention of acute liver injury^[50]. Our *in vitro* experiments showed that transfection of the EC-SOD plasmid in

Hep G2 and Hep3B cells led to an increase in SOD activity in cell culture medium. Transfected cells were more resistant to the exposure of superoxide anions generated by hypoxanthine and xanthine oxidase, and to HER by an HER-generating system. We employed polycationic liposomes to deliver the human EC-SOD gene via portal vein injection in mice^[50]. Two days after the gene delivery, a 55-fold increase in human EC-SOD gene expression was detected. When mice receiving the EC-SOD gene delivery were challenged with GalN and LPS, their serum ALT levels were much lower than those receiving control vector delivery, whereas their serum SOD levels were higher. Moreover, liver levels of the reduced form of glutathione (GSH) were preserved, and lipid peroxidation products, malondialdehyde (MDA) and 4-hydroxyalkenal (HAE) were reduced. Liver histology confirmed the effectiveness of the gene delivery. Thus, the overexpression of the human EC-SOD gene protected the mice from hepatotoxin-induced liver injury^[50]. Laukkaen *et al*^[51] achieved similar results when adenovirus-derived EC-SOD was delivered to prevent acetaminophen-induced acute liver injury in mice. Delivery of the Cu/Zn-SOD gene by adenoviral vector was also shown to protect rats from alcohol-induced liver injury^[52]. Cu/Zn-SOD was expressed in approximately 60% of liver cells with 3-5 fold higher levels of SOD compared to controls. The treatment of alcohol-fed rats with Cu/Zn-SOD gene delivery attenuated alcohol-induced elevation of serum aminotransferase levels with a 60% decrease of ALT levels and a 39% decrease in AST levels. Liver histopathology demonstrated less inflammation, reduced steatosis and no detectable necrosis. Free radical adduct formation measured by electron spin resonance (ESR) spectroscopy was reduced in mice treated with Ad.Cu/Zn-SOD. Nuclear extracts examined by electrophoretic mobility shift assays demonstrated a decrease in activation and translocation of NF- κ B. Thus, these studies provide substantial evidence that the delivery of SOD isoforms will protect the liver from acute and chronic liver injury in which the generation of ROS is a major component in the pathogenesis of the injury process.

Zhong *et al*^[53] evaluated adenoviral delivery of either Mn-SOD or Cu/Zn-SOD in a cholestatic model of liver injury. Rats were treated with AdMnSOD or AdCu/ZnSOD 3 d prior to bile duct ligation (BDL). Over 90% of hepatocytes were infected by virus. Mn-SOD and Cu/Zn-SOD activity increased 4-fold at d 3 and remained elevated up to 2-fold for 3 wk after the injection of adenoviruses. Elevated serum aminotransferase levels in rats with BDL were abrogated by 70% with AdMnSOD and 40% with AdCu/ZnSOD. Alkaline phosphatase (ALP) release was reduced by 50% with AdMnSOD and by 25% with AdCu/ZnSOD. AdMnSOD was also able to abrogate the increase in 4-hydroxynonenal (lipid peroxidation product). Enhanced NF- κ B activation, TGF- β and TNF- α expression in the cholestatic liver were reversed by AdMnSOD delivery. Furthermore, BDL caused enhanced fibrosis and a 20-fold increased level of procollagen type I (α 1) mRNA in the liver. These changes were reversed significantly by prior Ad-Mn-SOD gene delivery. Thus, it appears that antioxidant gene delivery is a promising

approach to prevent a variety of forms of liver injury, including drug-induced toxicity, ethanol consumption, and cholestasis or ischemia/reperfusion-associated liver injury^[54], as well as hepatic fibrogenesis^[53].

EXTRACELLULAR MATRIX REMODELING

ECM is composed of collagens, glycoproteins, proteoglycans, glycosaminoglycans, and other proteins^[55-56]. In the normal liver, collagens types I, III, V and XI are principally found in the capsule, around large vessels, and in the portal triad. Of note, the ECM components of the subendothelial space are typically low in collagen type I. Changes in the proportion of ECM components also contribute to the activation of HSC. Matrix metalloproteinases (MMPs) are important in the modeling and degradation of ECM, and they are classified according to their target substrates as interstitial collagenases, gelatinases, stromelysins, and metalloelastases^[57-58]. Stromelysin-1 degrades proteoglycans and glycoproteins. MMP-1 degrades collagen type I, while MMP-2 and MMP-9 degrade collagen type IV. These MMPs require activation by proteolytic cleavage and are inactivated by tissue inhibitors of metalloproteinases (TIMPs). The balance of production and degradation of ECM components maintains normal liver structure. The increased and disordered deposition of ECM components that occurs in fibrotic liver disease, especially fibrillar collagen types I and III, is due to unbalanced excessive production and reduced degradation. Enhancing the production of collagenase by gene delivery is a possible strategy to promote tissue remodeling and minimize the net accumulation of ECM components in chronic liver injury. Various collagenase genes have been delivered in attempts to decrease ECM deposition in animal models of hepatic fibrosis. Iimuro *et al.*^[59] evaluated the effectiveness of adenovirus-mediated gene delivery of matrix metalloproteinase-1 on the attenuation of liver fibrosis induced by thioacetamide or BDL in rats. The administration of Ad5MMP-1 significantly attenuated hepatic fibrosis with resolution within 2 wk, a decrease in cross-linked collagen type I, hydroxyproline content, and SMA-positive HSC, which corresponded to a decreased number of activated HSC in the liver sections. The treatment with Ad5MMP1 induced a transient increase in serum ALT levels that resolved by wk 4. This may reflect hepatocyte damage induced by adenoviral gene delivery. Subsequently, enhanced hepatocyte proliferation was found in the animals treated with MMP-1 gene therapy.

Siller-López *et al.*^[60] evaluated the efficacy of adenoviral delivery of MMP8 in ameliorating liver cirrhosis induced by either BDL or CCl₄ intoxication in rats. Marked expression of MMP-8 was found to reach a level of 550 pg/mL in AdMMP8-treated cirrhotic animals. This was accompanied with a significant decrease of a fibrotic score by 45% in BDL rats, as indicated by decreased liver hydroxyproline content. Moreover, TIMP-1, MMP-9, and HGF were found to be increased, whereas, TGF- β was decreased in animals receiving the MMP-8 gene delivery. The gene delivery also improved ascites, hepatic function tests, and gastric varices in these cirrhotic rats.

Thus, MMP-8 gene delivery seems to be highly effective in accelerating the resolution of the deposited ECM components in experimental animal models of hepatic fibrosis. However, immunoreaction to the adenoviral vectors or a high viral titer used, caused marked hepatocyte damage in those animals receiving adenoviral gene delivery^[60].

Urokinase-type plasminogen activator (uPA) is known to be an initiator of matrix proteolysis and promotes degradation of the extracellular matrix. Salgado *et al.*^[61] employed adenoviral vector to deliver human uPA in a rat model of hepatic fibrosis induced by chronic CCl₄ intoxication. At the end of 6 wk of repeated CCl₄ injections, rats were given Ad- Δ huPA via iliac vein injection. Animals were sacrificed 2 d after Ad- Δ huPA treatment for evaluating the gene delivery efficacy. Human uPA gene expression was detected in 46-80% of hepatocytes with marked elevation of human uPA in serum. The successful human uPA gene delivery resulted in an almost complete resolution of periportal and centrilobular fibrosis when compared to the animals with a control vector injection (Ad-GFP) as evidenced by histochemical staining for ECM components. The gene delivery also led to an elevated MMP-2 activity, and induced hepatocyte proliferation with increased levels of HGF. Thus, the study indicated that enhanced uPA expression by a gene delivery approach may activate MMP activity and accelerate ECM degradation, as well as facilitate a resolution of hepatic fibrosis. The same group performed another experiment in BDL rats and found that combination of adenoviral delivery of human uPA gene with biliodigestive anastomosis achieved a synergistic effect in reducing hepatic fibrosis^[62]. These studies provide substantial evidence that hepatic fibrosis is reversible in animal models when the underlying injury process is attenuated or stopped and an accelerated tissue remodeling occurs.

CONCLUSIONS AND PROSPECTS

New understanding and insights into the pathogenesis of hepatocellular damage and hepatic fibrogenesis, and the development of molecular techniques, such as new viral or non-viral vector systems, RNAi, ribozyme and antisense technologies have made it possible to modulate the expression of specific genes involved in the key pathways of liver injury and fibrosis. The fact that blocking Fas-ligand activity by siRNA or antisense against Fas receptor almost completely prevented Fas ligand-induced fulminant liver failure and animal mortality is noteworthy. It is also striking that silencing of a key enzyme, caspase 3 or 7, in an apoptotic cascade by siRNA blocks the cell damage. The transfer of anti-oxidant genes, such as isoforms of SOD, by viral or non-viral vectors prevents a variety of forms of liver injury in which ROS generation is involved in the pathogenic pathways. Alternatively, when the overexpression of fibrogenic cytokines, such as TGF- β and PDGF and their receptors are inhibited at the mRNA level with antisense, ribozymes or siRNA, or interrupted by specific antibodies or protein molecules, the interventions result in marked amelioration of hepatic

fibrosis. Moreover, delivery of collagenase genes by adenoviral vectors promotes the resolution of excessive deposition of ECM components and reverses hepatic fibrosis. Although these progresses in treatment of liver injury and fibrogenesis are encouraging, one issue that requires additional attention remains that targeting these molecular therapeutics to specific cell types (hepatocytes, Kupffer cells or HSC) is critical in avoiding undesired effects on other organs or cell types^[39,41,63].

Gene therapy itself is still in its infancy, and ideal gene delivery systems for selective gene transfer with high and prolonged gene expression, as well as less cytotoxicity or immunogenicity remain to be developed. Nevertheless, molecular therapeutics has already proven to be powerful research tools and explorative experimental medicine. They have demonstrated some promise as novel modalities in reducing liver injury, inhibiting HSC activation, and promoting the resolution of fibrotic deposition. However, the transition of promising molecular therapeutics from animal models to clinical trials for the validation of their clinical efficacy, adverse effects, target patient population will usually take a quite long period of time. One may anticipate that in combination with other more standard therapeutic modalities, these molecular approaches may yield effective treatments for hepatic injury and fibrogenesis in man in the foreseeable future.

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EDITORIAL

Progress in treatment of massive ascites and hepatorenal syndrome

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Abstract

Massive ascites and hepatorenal syndrome (HRS) are frequent complications of liver cirrhosis. Thus, effective therapy is of great clinical importance. This concise review provides an update of recent advances and new developments. Therapeutic paracentesis can be safely performed even in patients with severe coagulopathy. Selected patients with a refractory or recurrent ascites are good candidates for non-surgical portosystemic shunts (TIPS) and may have a survival benefit and improvement of quality of life. Novel pharmaceutical agents mobilizing free water (aquaretics) are currently under test for the therapeutic potential in patients with ascites. Prophylaxis of hepatorenal syndrome in patients with spontaneous bacterial peritonitis is recommended and should be considered in patients with alcoholic hepatitis. Liver transplantation is the best therapeutic option with long-term survival benefit for patients with HRS. To bridge the time until transplantation, TIPS or Terlipressin and albumin are good options. Albumin dialysis can not be recommended outside prospective trials.

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Key words: Albumin dialysis; Aquaretics; Free water clearance; Liver cirrhosis; Liver transplantation; Paracentesis; Pathophysiology; Portosystemic shunt; Spontaneous bacterial peritonitis; Terlipressin

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INTRODUCTION

Haemodynamic alterations and activation of neurohumoral systems are essential in the pathophysiology of ascites

formation^[1,2]. The most common circulatory alterations in patients with cirrhosis of the liver are portal hypertension and peripheral arterial vasodilatation which results in a decrease of centrally effective blood volume. As a consequence, neurohumoral systems are activated in an attempt to maintain intravascular volume (Figure 1). Among those, activation of the renin-angiotensin-aldosterone system, the sympathetic nervous system and the non-osmotic release of arginin-vasopressin play the major role. These neurohumoral systems induce renal sodium and water retention leading to the formation of ascites. The most severe form with renal vasoconstriction and a decrease of renal blood flow leads to hepatorenal syndrome. Recently, it has been suggested that a decrease in cardiac output may contribute to HRS^[3].

Novel therapeutic strategies for ascites and hepatorenal syndrome thus focus to counteract the initial changes of this pathophysiological cascade: controlled reduction of portal hypertension and/or peripheral vasoconstriction combined with plasma expanders.

TREATMENT OF MASSIVE ASCITES

In order to achieve fast relief, patients with massive ascites undergo therapeutic paracentesis^[4]. Apart from the risk of infection which can easily be prevented, major concerns have been raised regarding the risk of bleeding and haemodynamic instability. It has been shown that large volume paracentesis can be safely performed even in patients with severely impaired coagulation parameters^[5,6]. In a study investigating more than 500 patients with cirrhosis of the liver, an average paracentesis of 9 g/L was performed^[5]. Although the majority of patients exhibited less than 50×10^9 /L platelets and more than a quarter had severely prolonged prothrombin time, no case had severe complication or bleeding.

Following large volume paracentesis rapid re-formation of ascites may effectively reduce central blood volume and compromise systemic haemodynamic and renal function. To prevent intravenous administration of plasma expanders, a paracentesis has been established^[2,7]. For large volume paracentesis (more than 6 L) 20% human albumin in a concentration of at least 6 g per liter ascites seems to be the safest strategy. Recent work demonstrated that the incidence of paracentesis-induced circulatory dysfunction following paracentesis of less than 6 L is only 7% with albumin or saline as plasma expanders with almost no clinical complications^[8]. Thus, it may be safe and cost-effective to perform paracentesis of up to 6 L without albumin.

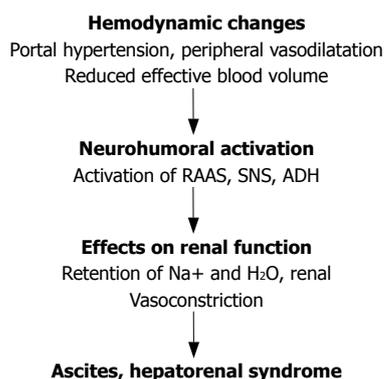


Figure 1 Pathogenesis of ascites formation and hepatorenal syndrome in patients with cirrhosis.

Table 1 Definition of refractory and recurrent ascites according to the consensus of the International Ascites Club^[9]

| | |
|--------------------|--|
| Refractory ascites | cannot be mobilized by diuretics because of a lack of response (mean weight loss less than 200g/d during the last 4 d) or the development of diuretic-induced complications such as hyponatremia, hypokalemia, renal impairment, hepatic encephalopathy, precluding an effective diuretic dosage |
| Recurrent ascites | recurs at least on 3 occasions within 1 year despite prescription of dietary sodium restriction and adequate diuretic dosage |

REFRACTORY AND RECURRENT ASCITES

Refractory and recurrent ascites have been defined according to a consensus conference of the International Ascites Club^[9] (Table 1). Repeated large volume paracentesis in addition to diuretic treatment is the standard treatment for these patients. Reduction of portal hypertension with non-surgical shunts (transjugular intrahepatic portosystemic shunt = TIPS) has the potential to markedly increase renal sodium excretion^[10]. This has prompted several prospective randomized controlled trials comparing repeated paracentesis with insertion of a TIPS (Table 2). The unequivocal result of all four trials^[11-14] has a highly significant advantage in the control of ascites by TIPS. Regarding survival, two trials have shown a significant advantage. Possibly, inclusion of patients with more severely impaired liver function and a bilirubin above 5mg/100mL at inclusion may be responsible for the lack of survival benefit following TIPS^[15] (Table 3). Encephalopathy following TIPS should be avoided as the major obstacle for improvement of quality of life as compared to paracentesis patients^[13]. Thus, careful selection of patients and management in experienced centers are the prerequisite for a TIPS benefit. Patients with ascites resolution following TIPS insertion enjoy a markedly improved quality of life^[16].

AQUARETICS

Vasopressin-V2-receptor antagonists mobilize free water and thus might be an excellent alternative or adjunct to

Table 2 Important features of large prospective trials^[11-14] comparing TIPS with paracentesis for massive ascites

| | Rossle ^[11] | Gines ^[12] | Sanyal ^[13] | Salerno ^[14] |
|-----------------------------|------------------------|-----------------------|------------------------|-------------------------|
| Patients/selected from pts. | 60/155 | 70/119 | 109/525 | 66/137 |
| Complete response (%) | 79 vs 24 | 51 vs 17 | 58 vs 16 | 61 vs 3 |
| Survival benefit of TIPS | yes | no | trend | yes |
| Number of centers | 2 | ≥5 | 6 | 3 |
| Child-Pugh C (%) | 38 | 37 | ? | 76 |
| Athyltox. Zirrhose (%) | 79 | 51 | 62 | 42 |
| Severe encephalop. (%) | 23 vs 13 | 60 vs 34 | 29 vs 18 | 61 vs 39 |
| Mean TIPS Ø (mm) | 9 | 8→10 | 10 | ? |

Table 3 Serum bilirubin concentration at study inclusion and during follow-up (µmol/L, mean±SD)

| | Rossle ^[11] | Gines ^[12] | Sanyal ^[13] | Salerno ^[14] |
|-----------------------------|------------------------|-----------------------|------------------------|-------------------------|
| Cut-off for study inclusion | 5 | 10 | 5 | 6 |
| Baseline | 1.7±0.2 | 2.0±0.2 | 1.9±0.2 | 1.6±0.1 |
| Follow-up(mo) | 2.9±0.9 | 4.6±2.0 | 2.2±2.1 | 2.1±0.2 |

Table 4 Definition of hepatorenal syndrome according to the consensus of the International Ascites Club^[9]

| |
|--|
| HRS Type 1: Rapidly progressing renal failure (<2 wk) ≥2-fold increase of serum creatinine to >221µmol/L or 50% decrease of creatinine clearance to <20mL/min |
| HRS Type 2: Not rapidly progressing renal failure |
| Serum creatinine >132.6 µmol/L or |
| Creatinine clearance <40mL/min |
| Absence of shock, ongoing bacterial infection, current or recent treatment with nephrotoxic drugs, gastrointestinal or renal fluid loss |
| No sustained improvement upon withdrawal of diuretics and plasma volume expansion |
| Proteinuria <0.5g/d, no abnormalities of renal ultrasound |

diuretic treatment. The efficacy and safety of these new compounds in patients with cirrhosis have recently been demonstrated^[17,18]. In these patients, urine volume increased and urine osmolarity dose-dependently decreased, thus demonstrating an increase of free water clearance. Moreover, while patients in the placebo group gained weight, body weight was stable with a lower dose and clearly decreased with a higher dose of the V2-receptor antagonist. This promising new pharmaceutical concept is currently under investigation in international phase II/III trials.

HEPATORENAL SYNDROME

According to established criteria, hepatorenal syndrome can be classified into type 1 and type 2^[9] (Table 4). Rapid progressive type 1 exhibits a very poor prognosis with a 3-month mortality rate of above 90%^[19]. Thus, prophylaxis of HRS is an important task and effective treatment is a highly desirable goal.

PROPHYLAXIS OF HRS

In patients with severe alcoholic hepatitis, the TNF-α inhibitor pentoxifyllin significantly reduces the incidence of

HRS, HRS-related and over-all mortality^[20]. Spontaneous bacterial peritonitis is often followed by deterioration of renal function and even hepatorenal syndrome. Interestingly, a randomized prospective trial^[21] showed that intravenous albumin administration (1.5g/kg per day on day one and 1g/kg per day on day three) together with antibiotic treatment with cefotaxim is clearly superior to antibiotic treatment alone because renal failure and moreover mortality were significantly reduced in hospital and during a 3-month follow-up period.

TREATMENT OF HEPATORENAL SYNDROME TYPE 1

Following liver transplantation, patients with HRS may reach 5-year survival probability of 60%^[22]. While this is a tremendous improvement compared to the spontaneous prognosis, survival rates are significantly lower than those in patients undergoing liver transplantation with normal renal function. Moreover, in many Western countries, waiting lists for liver transplantation are steadily growing, thus increasing the need to bridge severely sick patients to transplantation. Therapeutic concepts are needed to normalize renal function in patients with hepatorenal syndrome type 1. As stated in the introduction, very early interventions in the pathomechanistic cascade would seem more promising.

Indeed, controlled reduction of portal hypertension with TIPS seems to be rather effective. In an uncontrolled trial, the average mean survival time was around 4 mo in 14 out of 23 patients with HRS type 1 who received TIPS^[23]. Nine of the 23 patients, however, were at high risk for liver failure and therefore did not receive TIPS. For these severely ill patients, strategies should be used to counteract peripheral vasodilatation combined with volume expansion.

Several studies suggest that vasopressin analogues combined with albumin may be suitable to reverse hepatorenal syndrome^[24,26]. Terlipressin (average daily dose of 3 mg) could be a valuable option for patients with HRS type 1 and very poor liver function to bridge the time to transplantation if combined with plasma expanders^[27]. In this retrospective analysis best results with terlipressin were seen in patients with Child-Pugh score below 12 points, receiving at least 3 mg per day. However, only one randomized controlled trial on terlipressin has shown improvement of renal function in patients with HRS type 1 compared to no response in the placebo group^[28]. No survival data are provided.

In numerous uncontrolled observations extracorporeal albumin dialysis (MARS) has been suggested as a beneficial therapy for patients with acute-on-chronic liver failure. There are only two randomized trials investigating the effects of MARS, comprising a total of 23 patients with hepatorenal syndrome^[29,30]. No 30-d survival benefit has been demonstrated, disqualifying this procedure for the use outside of controlled trials^[30].

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EDITORIAL

Safety and clinical efficacy of granulocyte and monocyte adsorptive apheresis therapy for ulcerative colitis

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Abstract

Active ulcerative colitis (UC) is frequently associated with infiltration of a large number of leukocytes into the bowel mucosa. Therefore, removal of activated circulating leukocytes by apheresis has the potential for improving UC. In Japan, since April 2000, leukocytapheresis using Adacolumn has been approved as the treatment for active UC by the Ministry of Health and Welfare. The Adacolumn is an extracorporeal leukocyte apheresis device filled with cellulose acetate beads, and selectively adsorbs granulocytes and monocytes/macrophages. To assess the safety and clinical efficacy of granulocyte and monocyte adsorptive apheresis (GMCAP) for UC, we reviewed 10 open trials of the use of GMCAP to treat UC. One apheresis session (session time, 60 min) per week for five consecutive weeks (a total of five apheresis sessions) has been a standard protocol. Several studies used modified protocols with two sessions per week, with 90-min session, or with a total of 10 apheresis sessions. Typical adverse reactions were dizziness, nausea, headache, flushing, and fever. No serious adverse effects were reported during and after GMCAP therapy, and almost all the patients could complete the treatment course. GMCAP is safe and well-tolerated. In the majority of patients, GMCAP therapy achieved clinical remission or improvement. GMCAP is a useful alternative therapy for patients with steroid-refractory or -dependent UC. GMCAP should have the potential to allow tapering the dose of steroids, and is useful for shortening the time to remission and avoiding re-administration of steroids at the time of relapse. Furthermore, GMCAP may have efficacy as the first-line therapy for steroid-naive patients or patients who have the first attack of UC. However, most of the previous studies were uncontrolled trials. To assess a definite efficacy of GMCAP, randomized, double-blind, sham-controlled trials are necessary. A serious problem with GMCAP is cost; a single session costs ¥145 000 (\$1 300). However, if this treatment prevents hospital admission, re-administration of steroids and

surgery, and improves a quality of life of the patients, GMCAP may prove to be cost-effective.

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Key words: Clinical efficacy; Granulocyte and monocyte adsorptive apheresis; Leukocytapheresis; Safety; Ulcerative colitis

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INTRODUCTION

Ulcerative colitis (UC) is an inflammatory bowel disease of unknown etiology that involves colon and rectum. Active UC is frequently associated with infiltration of large number of leukocytes into the bowel mucosa^[1,2]. The infiltrated leukocytes can cause extensive mucosal tissue injury by releasing a large number of inflammatory mediators such as prostaglandins, leukotrienes, platelet activating factor, thromboxanes, oxygen radicals, proteases, and cytokines^[3]. Therefore, removal of activated circulating leukocytes by apheresis has the potential for improving the bowel inflammation and patient status in UC. Recently, leukocytapheresis has been tried as a novel approach for UC^[4,5].

Clinical efficacy and safety of leukocytapheresis for UC were initially investigated in a multicenter study in Japan, and the results were published in 1999^[5]. In that study, 120 patients were randomly divided into two groups; one group received leukocytapheresis and the other group conventional drugs (corticosteroids and/or sulfasalazine/mesalazine). Clinical efficacy was observed in 58% of patients in the leukocytapheresis group compared with 44% in the drug group. Adverse effects were noted in 8% of patients in the leukocytapheresis group compared with 43% in the drug group. From these data, leukocytapheresis seemed to be superior to conventional drugs, and this was more striking in patients with severe and intractable diseases^[5]. In Japan, since April 2000, leukocytapheresis using Adacolumn (Japan Immunoresearch Laboratories, Takasaki, Japan)^[6] has been approved as the treatment of patients with active UC by the Ministry of Health and

Table 1 Summary of trials of GMCAP therapy using Adacolumn for patients with active UC

| Authors (yr) | Indications for GMCAP | n | Apheresis protocol | Adverse effects (% of patients) | Efficacy (%) |
|---|---|---------|-------------------------------------|---------------------------------|--|
| Shimoyama <i>et al</i> ^[9] (2001) | Refractory to conventional drugs | 53 | Standard ¹ | 9% | Remission: 21% Improvement: 37% |
| Tomomasa <i>et al</i> ^[10] (2003) | Steroid-refractory children | 12 | 1 session/wk for 5-10 wk | 9% | Improvement: 67% |
| Hanai <i>et al</i> ^[11] (2003) | Steroid-refractory Steroid-naive | 31 8 | 10 or 11 sessions over 11 wk | 18% | Remission: Steroid-refractory 81% Steroid-naive 88% |
| Suzuki <i>et al</i> ^[12] (2004) | Steroid-naive | 20 | 2 sessions/wk for 3-5 wk | 10% | Improvement: Steroid-refractory 6% Steroid-naive 12% Remission: 85% |
| Naganuma <i>et al</i> ^[13] (2004) | Steroid-refractory Steroid-dependent | 44 | Standard ¹ | 5% | Remission: 55% Improvement: 20% |
| Hanai ^[14] (2004) ² | Steroid-dependent | 46 | 11 sessions over 10 wk | 22% | Remission: 83% |
| Yamamoto <i>et al</i> ^[15] (2004) | Mild-to-moderate active distal disease | 30 | Standard ¹ | 27% | Remission: 70% Improvement: 17% |
| Domenech <i>et al</i> ^[16] (2004) ³ | Steroid-dependent | 14 | Standard ¹ | 15% | Remission: 62% Improvement: 14% |
| Kanke <i>et al</i> ^[17] (2004) | Mild-to-severe disease | 60 | 10 sessions over 12 wk ⁴ | 18% | Remission: 23% Improvement: 60% |
| Kim <i>et al</i> ^[18] (2005) | Refractory to conventional drugs | 27 | Standard ¹ | 11% | Improvement: 70% |

GMCAP, granulocyte and monocyte/macrophage adsorptive apheresis; UC, ulcerative colitis.

¹Five apheresis sessions for five consecutive weeks; session time, 60 min; blood flow rate, 30 mL/min.

²This study was a randomized controlled trial comparing efficacy of GMCAP and prednisolone.

³This study also included 12 patients with Crohn's disease.

⁴One or two sessions per week; session time, 60 or 90 min.

Welfare. Hence, currently the Adacolumn is available in the market throughout Japan and is being used for treating UC. The Adacolumn is an extracorporeal leukocyte apheresis device filled with cellulose acetate beads, and selectively adsorbs granulocytes and monocytes/macrophages; lymphocytes are not significantly adsorbed^[7]. Thus, the Adacolumn is for selective granulocyte and monocyte/macrophage adsorptive apheresis (GMCAP). As leukocytapheresis therapy, Cellsorba (Asahi Kasei Medical Co., Ltd, Tokyo, Japan)^[8] is also available in Japan. The Cellsorba is filled with very fine polyester fiber as adsorptive carriers, and removes lymphocytes in addition to granulocytes and monocytes.

In this paper, we have discussed safety and efficacy of GMCAP using the Adacolumn, which has been used as leukocytapheresis therapy in our institution. Since 2000, there have been 10 open trials of the use of GMCAP to treat UC (Table 1)^[9-18]. Eight of the ten trials were performed in Japan. We mainly reviewed these trials to assess clinical efficacy and safety of GMCAP for UC. Finally, we discussed possible future role of GMCAP in the management of UC.

GMCAP procedures

The Adacolumn^[6] is a single use adsorptive type apheresis column, with a volume of 335 mL, filled with 220 g of cellulose acetate beads of 2 mm diameter as the column adsorptive carriers. An outline of GMCAP procedures using the Adacolumn is shown in Figure 1. GMCAP was

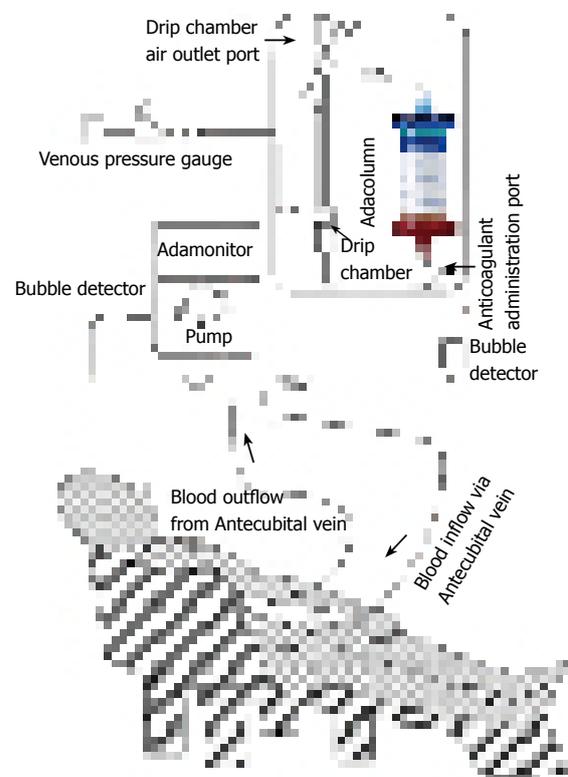


Figure 1 An outline of GMCAP procedures using the Adacolumn. Blood is drawn into the column from the antecubital vein of one hand, and returned to the patient via antecubital vein of the contralateral hand. GMCAP, granulocyte and monocyte/macrophage adsorptive apheresis.

performed in out-patient clinic for patients with mild symptoms, and patients with severe symptoms were hospitalized for GMCAP. The process of performing GMCAP is relatively simple. Prior to apheresis, the system is primed by saline-containing anticoagulant, nafamostat mesilate or heparin, and during apheresis saline containing those anticoagulants is continuously administered into the column. Blood is drawn into the column from the antecubital vein of one hand, and returned to the patient via antecubital vein of the contralateral hand, without using a shunt. The commonly used apheresis time and blood flow rate are 60 min and 30 mL/min, respectively. During these procedures, the carriers adsorb about 65% of granulocytes, 55% of monocytes and 2% of lymphocytes from the blood in the column^[7]. After completion of each apheresis session, the residual blood in the column and circuit lines is returned to the patient by infusing physiological saline at the blood inflow line. Vital signs are continuously monitored during the apheresis by dialysis staff. One apheresis session (session time, 60 min; blood flow rate, 30 mL/min) per week for five consecutive weeks (a total of five apheresis sessions) has been a standard protocol. Recently, other protocols with two sessions per week or a total of 10 apheresis sessions have been used for patients with severe UC. Furthermore, apheresis sessions longer than 60 min and apheresis with a higher blood flow rate have been tried for severe diseases. In several institutions, the efficacy of the protocol with a longer session time and higher blood flow rate is being compared with that of the standard protocol^[17].

Indications for GMCAP

According to the Guidelines of the Investigation and Research Committee of Inflammatory Bowel Disease of the Ministry of Health and Welfare of Japan, GMCAP has been mainly used for patients with steroid-refractory (no or little improvement after high dose steroid therapy) and steroid-dependent (inability to decrease the steroid dosage) moderate-to-severe UC. For the majority of patients, corticosteroids were given during GMCAP therapy as concomitant medications. In contrast, steroid-naïve patients with milder UC were also treated with GMCAP^[11,12,15].

Adverse effects

No serious adverse effects were reported during and after GMCAP therapy, and almost all patients could complete the treatment course. From the data in the previous trials^[9-18], adverse effects were observed in 5%-27% of patients (Table 1). Typical adverse reactions were dizziness, nausea, headache, flushing, and fever. These symptoms lasted from only a couple of minutes to a couple of hours. Fever could be treated with antifebrile, and headache could be prevented with painkillers before the apheresis. Infectious problems due to GMCAP were rarely reported^[16]. In our study, one patient developed a mild liver dysfunction probably due to an anticoagulant, nafamostat mesilate^[15]. Thereafter, heparin was used instead. The liver dysfunction normalized without any special treatment.

Clinical efficacy

Patients were followed up regularly during and after GMCAP therapy, and the final efficacy assessment was performed at one or two weeks after the last apheresis session^[9-18]. The efficacy of GMCAP was evaluated using clinical activity index or disease activity index scores^[19-21]. In a prospective multicenter trial in Japan^[9], 53 patients refractory to conventional drug therapy were treated with the standard GMCAP protocol (five apheresis sessions for five consecutive weeks; session time, 60 min; blood flow rate, 30 mL/min) in combination with prednisolone. After the treatment, 21% and 37% of patients achieved remission and improvement (without remission), respectively, and the mean daily dose of prednisolone per patient was reduced from 24.4 mg at enrollment to 14.2 mg after GMCAP therapy. In another prospective multicenter study in Korea^[18], 27 patients with moderate-to-severe active UC refractory to conventional drugs were treated with the standard GMCAP protocol. Clinical improvement was observed in 70% of patients (44% markedly improved), and tapering down or discontinuation of steroids was possible in 56% of concomitant steroid users. Naganuma *et al*^[13] treated 44 steroid-refractory or -dependent patients with the standard GMCAP protocol. Twenty-four patients (55%) obtained remission, 9 (20%) showed a clinical response (without remission), and 11 (25%) remained unchanged. Only 20% of patients with severe steroid-refractory UC achieved remission, whereas 70% of patients with moderate steroid-refractory UC achieved remission. In 90% of patients with steroid-dependent UC, the daily dose of corticosteroids could be tapered during GMCAP therapy. In the long-term, 61% of patients who achieved clinical improvement or remission maintained remission, whereas 39% had relapsed. Approximately half of the relapsed patients were successfully treated with repeat GMCAP therapy. The Spanish Group for the Study of Crohn's Disease and Ulcerative Colitis (GETECCU)^[16] conducted a prospective, open, pilot study including 26 patients with steroid-dependent inflammatory bowel disease (UC, 14; Crohn's disease, 12). Patients were started on 60 mg/d of prednisone; after one week, GMCAP therapy with the standard protocol was started. The steroid dose was tapered weekly if there was clinical improvement. Remission was achieved in 62% and 70% of patients with UC and Crohn's disease, respectively. During a median follow-up of 12.6 mo, 6 of 8 UC patients maintained their clinical remission; however, only one Crohn's disease patient remained in remission after the first 6 mo of follow-up.

Higher remission or improvement rates have been reported using the modified GMCAP protocols. Hanai *et al*^[11] examined 31 patients with steroid-refractory UC and eight steroid-naïve patients who were treated with a total of 10 or 11 GMCAP sessions (session time, 60 min; blood flow rate, 30 mL/min); for patients with severe diseases, two sessions per week for the first three weeks and then weekly session for five weeks; for the other patients, weekly session for the first five weeks and after an interval of one week, weekly session for another five weeks. After the treatment, 81% of steroid-refractory and 88% of

steroid-naïve patients achieved remission, and 79% of patients maintained their remission during 12 mo. The same research group^[14] conducted a randomized controlled trial comparing the efficacy of GMCAP and prednisolone. Sixty-nine steroid-dependent patients were randomly assigned to two groups; 46 patients were given 11 GMCAP sessions (session time, 60 min; flow rate, 30 mL/min) over 10 wk in combination with prednisolone (GMCAP group), and the other 23 patients were treated with 30 mg/d of prednisolone (prednisolone group). Prednisolone was tapered or discontinued with improvement of disease in both the groups. At 12 wk after the treatment, 83% of patients in the GMCAP group and 65% in the prednisolone group achieved remission and the difference was not statistically significant. During the 12 wk of the treatment, the cumulative amount of prednisolone received per patient was 1 157 mg in the GMCAP group, which was significantly lower than 1 938 mg in the prednisolone group. Kanke *et al.*^[17] treated 60 patients with active UC with 10 GMCAP sessions with different duration (60 or 90 min) and frequency (one or two sessions per week) of apheresis. There was an association between the final disease activity and the duration of apheresis, and twice a week was superior to one session per week for the improvement of disease activity. Furthermore, twice a week or 90 min was superior to one session per week or 60 min for improvement of clinical symptoms such as diarrhea, abdominal pain, and bloody stools.

GMCAP has been also used for steroid-naïve patients or patients with milder UC^[12,15]. Suzuki *et al.*^[12] treated 20 steroid-naïve patients with active UC with 6-10 GMCAP sessions (two sessions per week; session time, 60 min; blood flow rate, 30 mL/min). Seventeen patients (85%) achieved remission, and 60% had maintained their remission during eight months. In our study^[15], GMCAP therapy with the standard protocol (no concomitant steroid medications) was performed for 30 patients with mild-to-moderately active distal UC. Clinical symptoms significantly improved after the third apheresis session. Clinical remission was achieved in 21 patients (70%), and clinical improvement (without remission) was recognized in 5 patients (17%). In 4 patients (13%), clinical response was not recognized. GMCAP was not effective for patients with long disease duration and those who had been treated with high-dose steroids. All of the 21 patients who achieved clinical remission were able to maintain remission during the 12-wk follow-up after GMCAP. The efficacy of GMCAP for pediatric UC has been reported. Tomomasa *et al.*^[10] retrospectively reviewed 12 steroid-refractory children who were treated with weekly GMCAP for 5-10 consecutive weeks. In eight patients, clinical symptoms improved after two apheresis sessions. The dose of steroid was tapered during GMCAP therapy by 50%. Four of the eight patients relapsed 3.5 mo after the last apheresis session, the other four patients remained in remission up to 22.8 mo.

DISCUSSION

A lot of research is going on to clarify the detailed mechanism of GMCAP on UC^[7,22-29]. Although during GMCAP,

the carriers adsorb 65% of granulocytes, 55% of monocytes and 2% of lymphocytes from the blood in the column, the number of these leukocytes in the systemic circulation did not fall immediately after the apheresis^[7]. This can be explained by an influx of new leukocytes into the systemic circulation from the marginal pools, principally the bone marrow during apheresis. Thus, the efficacy of GMCAP cannot be fully explained on the basis of reducing leukocytes. Rembacken *et al.*^[4] found that the markedly increased expression of $\alpha(m)$ integrin/Mac-1 and low L-selectin expression alter the capability of granulocytes to migrate to inflammatory sites. Kashiwagi *et al.*^[23] found a significant suppression of proinflammatory cytokines (interleukin [IL]-1 β , IL-6, IL-8 and tumor necrosis factor [TNF]- α production) by leukocytes, neutrophils chemotaxis, down-regulation of leukocyte adhesion molecule (L-selectin) and neutrophil adhesion to IL-1 β -activated endothelial cells after GMCAP for UC. Furthermore, the number of CD10-negative premature granulocytes increased, indicating increased turnover of these cells in the systemic circulation. Takeda *et al.*^[27] found that granulocyte adsorption to cellulose acetate beads required plasma IgG, the complement C3 and was inhibited by an antibody to leukocytes CD18. Furthermore, hepatocyte growth factor and IL-1 receptor antagonist (IL-1ra) which have strong anti-inflammatory actions were released by granulocytes that adhered to cellulose acetate beads. Hanai *et al.*^[28] found that TNF- α receptors I and II were significantly increased in the peripheral blood after apheresis. These observations suggest that selective granulocyte and monocyte adsorption is associated with modified peripheral blood leukocyte function favorable to patients with UC which reflect leukocyte hyperactivity. Further investigations are necessary to explain the precise mechanism of GMCAP.

No serious adverse effects were reported during and after GMCAP therapy, and almost all patients could complete the treatment course^[9-18]. GMCAP is fairly safe and well tolerated. The clinical remission and improvement rates were different among the studies because many factors such as the indications of GMCAP, disease activity before GMCAP, protocol of GMCAP (frequency, duration and a total number of apheresis), concomitant medications, and the method of efficacy assessment were different. However, the data from the previous trials suggest that GMCAP is a useful alternative therapy for patients with steroid-refractory or -dependent UC. GMCAP should have the potential to allow tapering the dose of steroids, and is useful for shortening the time to remission and avoiding re-administration of steroids at the time of relapse. Furthermore, GMCAP may have efficacy as the first-line therapy for steroid-naïve patients or patients who have the first attack of UC.

The modified apheresis protocols with two sessions per week, with 90-min session, or a total of 10 apheresis sessions seem to be more effective than the standard protocol (weekly, 60-min apheresis and a total of five sessions). The modified protocol should be used for patients with more severe diseases, and patients with milder diseases can be treated with the standard protocol. However, the most appropriate frequency and duration of apheresis, and number of apheresis sessions still remain

unknown. Although several patients maintained their remission after GMCAP therapy, the long-term efficacy of GMCAP should be assessed in future. Furthermore, whether GMCAP is useful as maintenance therapy during remission needs to be assessed. Most of the previous studies were uncontrolled trials; only one study was a randomized, open-labeled, controlled trial comparing GMCAP and prednisolone^[14]. To assess a definite efficacy of GMCAP, well-designed randomized controlled trials are necessary. In the United States and Canada, a randomized, prospective, double-blinded, placebo-controlled (sham-controlled) study to evaluate the safety and effectiveness of the Adacolumn apheresis system for the treatment of moderate-to-severe UC is being conducted^[30].

There have been few studies which examined the efficacy of GMCAP for Crohn's disease^[16,31-34]. One study reported that a five-session protocol of GMCAP seemed to be efficient in the treatment of steroid-dependent UC, but not in Crohn's disease^[16]. In contrast, several studies reported that GMCAP was useful to decrease the disease activity in patients with Crohn's disease^[31-34]. Kusaka *et al*^[33] treated six patients with active Crohn's disease unresponsive to conventional medications with GMCAP (five apheresis sessions for five consecutive weeks). The Crohn's disease activity index (CDAI) significantly decreased after GMCAP therapy. Three of the six patients responded to the therapy, and one patient could be induced to remission. Fukuda *et al*^[34] treated 21 patients with a CDAI of >200 and unresponsive to standard medication with GMCAP (five apheresis sessions for five consecutive weeks). After GMCAP therapy, significant improvements in CDAI scores were observed. GMCAP could be effective for inducing remission and improving the quality of life in patients with active Crohn's disease that is refractory to conventional therapy. However, further clinical studies are necessary to assess the efficacy of leukocytapheresis for Crohn's disease because the number of patients included in the previous studies was quite small.

A serious problem with GMCAP is cost; a single session of GMCAP costs ¥145 000 (\$1 300). Because of high cost, GMCAP therapy may not be favorable as a first-line alternative to steroids in patients with active UC. Up to now, the most common medication for UC has been steroids^[35,36]. When the patient does not respond to steroids and the disease is progressing, surgery is implemented as the last option. Most patients experience adverse effects associated with high dose and long-term administration of steroids and hence, they could have a poor quality of life^[35,36]. Patients with active UC can be successfully treated with leukocytapheresis, without the usage of corticosteroids, which is good news for both patients and physicians because the prolonged use of steroids may be limited and patient compliance diminished by potential adverse effects. If GMCAP prevents hospital admission, re-administration of steroids and surgery, and it improves and maintains a quality of life of the patients, GMCAP may prove to be cost-effective. The future of GMCAP in the treatment of UC requires further evaluation,

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Cardiopulmonary complications in chronic liver disease

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Abstract

Patients with cirrhosis and portal hypertension exhibit characteristic cardiovascular and pulmonary hemodynamic changes. A vasodilatory state and a hyperdynamic circulation affecting the cardiac and pulmonary functions dominate the circulation. The recently defined cirrhotic cardiomyopathy may affect systolic and diastolic functions, and imply electromechanical abnormalities. In addition, the baroreceptor function and regulation of the circulatory homeostasis is impaired. Pulmonary dysfunction involves diffusing abnormalities with the development of the hepatopulmonary syndrome and portopulmonary hypertension in some patients. Recent research has focused on the assertion that the hemodynamic and neurohumoral dysregulation are of major importance for the development of the cardiovascular and pulmonary complications in cirrhosis. This aspect is important to take into account in the management of these patients.

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Key words: Cirrhosis; Portal hypertension; Cardiomyopathy; Hemodynamics; Vasoactive substances; Baroreceptors; Hepatopulmonary syndrome; Portopulmonary hypertension; Autonomic dysfunction

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INTRODUCTION

The clinical picture of patients with cirrhosis is dominated by the classical complications to portal hypertension, such as ascites, bleeding from esophageal varices, and encephalopathy. In addition, a considerable number of patients show signs of peripheral vasodilatation with

palmar erythema and reddish skin, raised and bounding pulse, and a low systemic blood pressure indicating a hyperdynamic circulation^[1]. The hyperdynamic syndrome comprises an increased heart rate, cardiac output, and plasma volume, and a reduced systemic vascular resistance and arterial blood pressure^[2-6]. The typical circulatory changes in patients with cirrhosis are summarized in Table 1. Increased cardiac output in cirrhosis was described more than 50 years ago^[7] and a hyperdynamic, hyporeactive circulation is today a well-characterized element in the clinical appearance of these patients^[8]. In addition, patients with cirrhosis develop complications from a variety of organs including the heart, lungs, and kidneys, and other organ systems. Besides the hepatorenal syndrome, this has led to the introduction of new clinical entities, such as cirrhotic cardiomyopathy and the hepatopulmonary syndrome.

Among the mechanisms involved in the peripheral arterial vasodilatation in cirrhosis, intensive research has focused on the presence of arteriovenous communications, increased blood volume, and potent vasodilating systems such as the NO and endothelin (ET) systems^[9-14]. Activation of a number of neurohumoral homeostatic system, like the renin-angiotensin-aldosterone systems (RAAS), the sympathetic nervous system (SNS), and the hypothalamic/neuropituitary release of vasopressin also seem to play a pivotal role in the circulatory dysfunction in cirrhosis^[15-18].

This review will focus on the pathophysiological aspects of the systemic circulatory abnormalities in cirrhosis with emphasis on cardiac and pulmonary dysfunctions.

SYSTEMIC CIRCULATION IN CIRRHOSIS

The cardiac output is primarily determined by the venous return, heart rate, and myocardial contractility, all of which are controlled by the autonomic nervous system. Among the mechanisms that may raise the cardiac output are increased sympathetic nervous activity, vasodilatation (low systemic vascular resistance), increased blood volume, and the presence of arterio-venous communications. It is noteworthy that the majority of these physiological changes are present in cirrhosis and may more or less contribute to raise cardiac output^[1,19]. In the early stages of compensated cirrhosis, the presence of a hyperdynamic circulation is often not apparent. But with the progression of the liver disease, there is an overall association between the severity of cirrhosis and the degree of hyperdynamic circulation. Investigations on circulatory changes and reactivity from the upright to the supine position, and vice versa, suggest that the patients are mostly hyperdynamic in the supine position^[20,21]. On the other hand, the pressor systems are relatively deactivated in the supine position and

Table 1 Circulatory changes in patients with cirrhosis

| |
|--|
| Systemic circulation |
| Plasma volume ↑ |
| Total blood volume ↑ |
| Non-central blood volume ↑ |
| Central and arterial blood volume →↓(↑) |
| Cardiac output (→)↑ |
| Arterial blood pressure →↓ |
| Heart rate ↑ |
| Systemic vascular resistance ↓ |
| Heart |
| Left atrial volume ↑ |
| Left ventricular volume →(↓) |
| Right atrial volume→↑↓ |
| Right ventricular volume →↑↓ |
| Right atrial pressure →↑ |
| Right ventricular end-diastolic pressure → |
| Pulmonary artery pressure →↑ |
| Pulmonary capillary wedge pressure → |
| Left ventricular end-diastolic pressure → |
| Pulmonary circulation |
| Pulmonary blood flow ↑ |
| Pulmonary vascular resistance ↓(↑) |
| Renal circulation |
| Renal blood flow ↓ |
| Renal vascular resistance ↑ |
| Cerebral circulation |
| Cerebral blood flow ↓ → |
| Cutaneous and skeletal muscle circulation |
| Cutaneous blood flow →↑ |
| Skeletal muscular blood flow →↑ |

↑ Increase, → No change, ↓ Decrease.

it is well documented that sodium-water excretion is higher in the supine position than in the upright position^[22].

Pathophysiology of arterial vasodilatation

Peripheral vasodilatation in cirrhosis may be brought about either by overproduction of circulating vasodilators, or by vasodilators of intestinal or systemic origin, or by vasodilators that escape degradation in the diseased liver or bypass the liver through portosystemic collaterals^[19]. A predominantly splanchnic vasodilatation precedes renal sodium and water retention and plasma volume expansion, which correlates with activated counter regulatory vasoconstrictor systems^[6,19]. In 1988, Schrier *et al* proposed the "peripheral arterial vasodilatation hypothesis"^[23]. According to this theory, primary splanchnic arteriolar vasodilatation leads to the reduction of the overall systemic vascular resistance, to avoid arterial underfilling with low arterial blood pressure. A reduced effective blood volume, which is that part of the blood volume where baroreceptors are located, leads to the activation of vasoconstrictor systems and secondary sodium-water retention^[6,23-25]. Thus, most of the hemodynamic changes seen in cirrhosis can be explained by this theory, as shown in Figure 1.

Circulating vasodilators

In recent years, research has focused especially on vasodilating substances, such as NO, calcitonin gene-related peptide (CGRP), and adrenomedullin, but other vasodilators substances, like natriuretic peptides, tumor necrosis factor alpha, interleukins, substance P, ETs, and endocannabinoids,

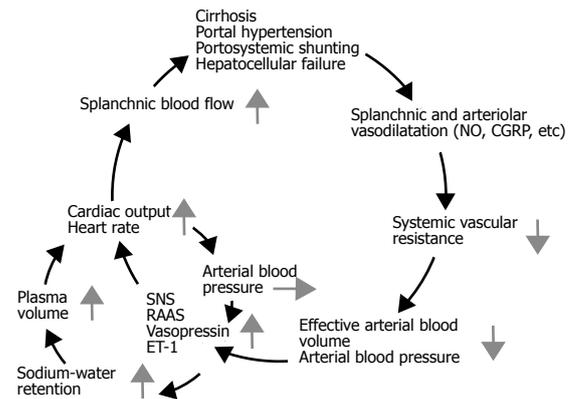


Figure 1 Pathophysiology of the splanchnic and peripheral arteriolar vasodilatation and systemic hemodynamic changes in cirrhosis. Endogenous vasodilators may escape hepatic degradation, owing to portosystemic shunting and/or hepatocellular damage, and induce vasodilatation preferentially in the splanchnic vascular area. Reduced systemic and splanchnic vascular resistance leads to a reduced effective arterial blood volume, and hence to activation of vasoconstrictor systems. The hemodynamic and clinical consequences are increases in cardiac output, heart rate, and plasma volume and decreased renal blood flow, low arterial blood pressure, and fluid and water retention. The development of the hyperdynamic circulation may increase portal inflow and further aggravate the portal pressure in a vicious cycle. SNS, sympathetic nervous system; RAAS, renin-angiotensin-aldosterone system; ET-1, endothelin-1.

have also been implicated^[12,26-32].

NO is synthesized in the vascular endothelium from L-arginine by NO synthase (NOS)^[33], of which three isoforms have been identified: inducible NOS (iNOS), constitutive endothelial NOS (ecNOS), and neuronal NOS (ncNOS)^[12,34]. In portal hypertension, there seems to be a diminished release of NO from sinusoidal endothelial cells in the cirrhotic liver^[34,35] whereas, in the systemic circulation, there is evidence of increased eNOS upregulation, which is probably related to shear stress^[26,33,36]. Exhaled air from cirrhotic patients contains higher NO levels than that of controls and correlates with the severity of disease and degree of hyperdynamic circulation; in animal models and cirrhotic patients, blockade of NO formation significantly increases arterial blood pressure and decreases plasma volume and sodium retention^[37-40]. Taken together, there is a growing body of evidence that the systemic NO production is increased and precedes the development of the hyperdynamic circulation in cirrhosis, thereby playing a major role in the arteriolar and splanchnic vasodilatation and vascular hyporeactivity^[12,41]. In addition, vascular endothelial growth factor (VEGF) seems to stimulate angiogenesis and the development of portosystemic collaterals, and recently blockade of the VEGF receptor-2 has been shown to inhibit this process^[42]. In spite of the experimental nature of the study, this principle may have some therapeutic implications in the treatment of portal hypertension.

CGRP, a 37-amino-acid neuropeptide with a neurotransmitter function, is on a molar basis the most powerful vasodilating peptide known^[43]. It is elevated in cirrhosis, especially in those patients with ascites and the hepatorenal syndrome^[43,44] and correlates to hemodynamic markers of vasodilatation and central hypovolaemia, such as cardiac output, systemic vascular resistance, arterial compliance, and central blood volume^[27,45-47]. Adrenomedullin is a vasodilating peptide with a sequence

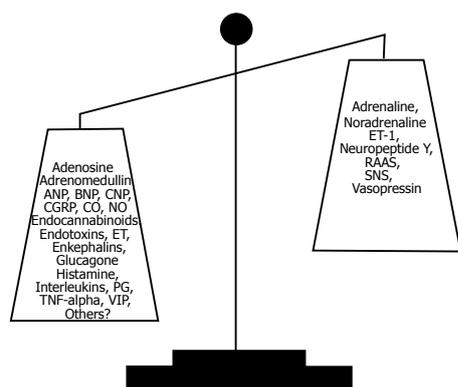


Figure 2 Imbalance between vasoconstricting and vasodilating forces. ANP, atrial natriuretic peptide; BNP and CNP: B- and C-type natriuretic peptides; CGRP, calcitonin gene-related peptide; ET, endothelin; TNF- α , tumor necrosis factor α ; VIP, vasoactive intestinal polypeptide; RAAS, The renin-angiotensin-aldosterone system; SNS, The sympathetic nervous system.

similarity to CGRP, it is primarily released from the adrenal medulla and induces relaxation of smooth muscle cells^[48]. The circulating levels of adrenomedullin seem to be higher in decompensated patients with cirrhosis and correlate with pressor substances, such as endothelin, renin, vasopressin, and catecholamines^[29,49,50].

In addition to a surplus of vasodilators, resistance to pressor hormones may play a role in the pathogenesis of vasodilatation, as patients with cirrhosis are hyporesponsive to the pressor effects of such potent vasopressors as noradrenaline, angiotensin II, and vasopressin^[51-53]. This may be brought about by a change in receptor affinity, a decrease in the numbers of receptors, and a variety of post-receptor defects^[54,55]. Helmy *et al* have reported hyporesponsiveness to angiotensin II and endothelin-1, chiefly because of enhanced NO generation^[41,56]. Thus, the excess of vasodilators combined with an inadequate hemodynamic response to vasoconstrictors may explain the vasodilatory state and vascular hyporeactivity in cirrhosis.

Vascular territories with vasoconstriction

Although cardiac output is increased, thus reflecting substantial vasodilatation, it may cover perfusions from vascular beds that are hyperperfused, normoperfused, and hypoperfused. The kidney in cirrhosis is a vascular region where vasoconstriction prevails and plays a pivotal role in the development of hepatic nephropathy with a decreased renal blood flow and glomerular filtration rate, increased sodium reabsorption, and decreased free water excretion^[6,57]. Liver dysfunction, central hypovolaemia, arterial hypotension, and neurohumoral activation with renal vasoconstriction seem to be of major importance. Recent results suggest that a decrease in the cardiac output in the face of severe vasodilatation and activation of the RAAS is an important determinant of the hepatorenal syndrome^[58]. Splanchnic vasodilatation and central hypovolaemia activate the SNS, the RAAS, the endothelin system, and increased release of vasopressin^[17,18]. The imbalance between vasoconstricting and vasodilating forces is illustrated in Figure 2. Secondary hyperaldosteronism and increased tubular sensitivity to aldosterone increase sodium reabsorption in the distal nephron, whereas SNS

stimulates sodium reabsorption in proximal tubules, the loop of Henle, and distal tubules^[59]. Angiotensin II mainly acts on the efferent arteriole and a low dose of an ACE-inhibitor may induce a significant reduction in glomerular filtration and a further reduction in sodium excretion, even in the absence of a change in arterial blood pressure. This suggests that the integrity of the RAAS is important for the maintenance of renal function in cirrhotic patients, and that RAAS overactivity does not solely contribute to the adverse renal vasoconstriction. Because of the arterial vasodilatation, the renal perfusion pressure is low and critically dependent on counter regulatory systems. For these reasons, blockers of these systems by ACE-inhibitors (captopril), angiotensin II antagonists (Losartan), β -adrenergic blockers, and V1 vasopressin antagonist may decrease further the renal blood flow^[60,61]. The hepatorenal syndrome denotes a functional and reversible impairment of renal function with a poor prognosis in patients with severe cirrhosis. Treatment is directed towards improving liver function, arterial hypotension, and central hypovolaemia, and reducing renal vasoconstriction for instance with the combined use of splanchnic vasoconstrictors such as terlipressin and plasma expanders like human albumin^[62].

Distribution of volumes and flow

Blood and plasma volumes are increased in patients with advanced cirrhosis^[25,63], and the distribution of blood in the different vascular beds is abnormal and relates to the severity of the disease^[64]. By different techniques it has been established that the central and arterial blood volume is most often decreased, whereas the non-central blood volume, in particular the splanchnic blood volume is increased in animals and patients with cirrhosis^[65-67]. The effective arterial blood volume is decreased with relation to the systemic circulatory derangement. Moreover, the central circulation time (i.e. central blood volume relative to CO) is substantially reduced and has a significant relation to poorer survival in advanced cirrhosis^[68,69].

During volume expansion, most cirrhotic patients respond with a further reduction in systemic vascular resistance rather than an increase in arterial blood pressure^[70,71]. Volume expansion by head-out water immersion provides, in principle, the same volume changes by central relocation as in healthy subjects^[70]. However, especially in decompensated cirrhosis, there may be a further decrease in the arterial blood pressure, owing to the unloading of baroreceptors, and renal salt-water excretion is prolonged and incomplete. The infusion of hyperosmotic solutions or albumin in cirrhosis results initially in a shift of fluid from the interstitial space into the plasma volume, with an expansion of the latter^[63,71]. Albumin infusion is important in the prevention of postparacentesis circulating dysfunction^[72]. When considering volume expansion in terms of the severity of the disease, certain differences become clear. Irrespective of severity, volume expansion produces a rise in stroke volume and cardiac output. Whereas in early cirrhosis there is a proportional expansion of the central and non-central parts of the blood volume, in late cirrhosis expansion is mainly confined to the non-central part, with a proportionally smaller increase in cardiac output, probably because of cardiac dysfunction (cirrhotic

cardiomyopathy) and abnormal vascular compliance^[71,73].

The increased plasma volume in cirrhosis should be considered secondary to the activation of neurohumoral mechanisms consequent on arterial vasodilatation, low arterial blood pressure, and reduced central and arterial blood volume. However, a non-volume-dependent activation of the SNS through hepatic reflexes, owing to portal hypertension, may occur. This has been documented in animal experiments and there are indications of such a reflex in men^[74]. Although the relative importance of non-volume-dependent sympathetic activation and volume/arterial pressure-dependent activation of SNS and other neurohumoral systems has not been finally established, the latter is probably far the most important.

CARDIAC DYSFUNCTION IN CIRRHOSIS

The hyperdynamic circulation in cirrhosis comprises increased cardiac output and work^[5,75]. In other circumstances, this would cause cardiac failure, but because of the decreased afterload as reflected by reduced systemic vascular resistance and increased arterial compliance, a left ventricular failure may be latent in cirrhosis^[75,76]. Cardiac failure may become manifest under strain or treatment with vasoconstrictors. This type of cardiac dysfunction has been termed as cirrhotic cardiomyopathy and includes impaired cardiac contractility with a systolic dysfunction, diastolic dysfunction, and electromechanical abnormalities with a prolonged *Q-T* interval^[77]. Various electrophysiological mechanisms for the conductance abnormalities and impaired cardiac contractility have been put forward, including reduced beta-adrenoceptor density, post-receptor signal defects, abnormal excitation-contraction coupling, and molecular abnormalities^[73].

Systolic dysfunction

At rest, when the systemic vascular resistance (afterload) against which the heart works is reduced, cardiac pressures are almost normal and may thereby mask an underlying ventricular dysfunction. Cardiac failure, therefore, becomes manifest only under conditions of hemodynamic stress. Thus, after the exercise, the left ventricular end-diastolic pressure increases, but the expected increase in cardiac stroke index and left ventricular ejection fraction (LVEF) are absent or subnormal, which indicates inadequate ventricular reserve response to a rise in ventricular filling pressure^[78,79]. A vasoconstrictor-induced increase in left ventricular afterload by 30% results in an increase in pulmonary capillary wedged pressure about double, without any change in the cardiac output^[80]. A similar pattern is seen after the insertion of transjugular intrahepatic portosystemic shunt (TIPS), but the increased cardiac pressures tend to normalize with time^[81-83]. A failure to increase cardiac output, despite an increased ventricular filling pressure, indicates that normalization of the afterload impairs cardiac performance and unmasks left ventricular dysfunction^[80]. Similar effects are seen after infusion of plasma expanders. Infusion of a plasma protein solution, however, increases cardiac output, as well as the right atrial pressure, pulmonary arterial pressure, and pulmonary capillary wedged pressure, whereas infusion of

packed red blood cells may not change these variables^[84].

The LVEF, i.e. the stroke volume relative to the left ventricular end-diastolic volume, is an often used measure of systolic function even though it is still very much influenced by preload and afterload. It has been reported to be normal at rest in some studies^[78,85-90] and reduced in one study in a subgroup of patients with ascites^[91]. The maximum aerobic exercise capacity and maximum heart rate are lower in the majority of patients with cirrhosis^[79,85,92]. After exercise, LVEF increases significantly less in cirrhotic patients than in controls^[79,92,93]. The reduced functional capacity may be attributed to a combination of blunted heart rate response to exercise, reduced myocardial reserve, and profound skeletal muscle wasting with impaired oxygen extraction^[85,94]. Normalization of the low systemic vascular resistance by vasoconstrictors results in an increase in the left atrial and left ventricular filling pressures^[75,80]. Therefore, attempts to normalize the reduced cardiac afterload seem to unmask a latent ventricular dysfunction which appears to be resistant to inotropic drugs^[77,80]. The expanded blood volume in advanced cirrhosis contributes to a persistent increase in cardiac output, which may overload the heart, with impaired cardiac contractility as the outcome^[20,86,95]. In patients with advanced cirrhosis and severe vasodilatation, activation of the RAAS, and impaired renal function, a reduced systolic function (a decrease in cardiac output) seems to be a major determinant for the development of the hepatorenal syndrome^[58].

Diastolic dysfunction

A diastolic dysfunction implies changes in myocardial properties that affect left ventricle acceptance of a sufficient volume during the diastole, despite normal filling pressures; the increased stiffness of the myocardial wall will thus result in impaired filling of the left ventricle^[96]. This increases the transmitral pressure gradient and the atrial contribution to ventricular filling in order to normalize the left ventricular diastolic volume. Determinants of a diastolic dysfunction include impaired left ventricular diastolic filling, in spite of high stroke volume. In the Doppler-echocardiogram, the E-wave reflects the early rapid transmitral flow and the A-wave the late atrial contribution to filling. Indications of diastolic dysfunction are a decreased E/A ratio and delayed early diastolic transmitral filling with prolonged deceleration and isovolumetric relaxation times^[97], as shown in Figure 3. In cirrhosis, the morphological basis of cirrhotic cardiomyopathy seems to be cardiac hypertrophy, patchy fibrosis, and subendothelial edema^[77,93]. In a number of studies, A-wave and E-wave velocities and deceleration times are much increased and the E/A-ratio is decreased in cirrhotic patients, especially in those with ascites^[91,97]. Recent studies of ventricular diastolic filling in cirrhosis support the presence of a subclinical myocardial disease with diastolic dysfunction, which, in ascitic patients, is improved after paracentesis and aggravated after TIPS^[82,88,91,97]. In these decompensated patients, paracentesis seems to ameliorate diastolic, but not systolic, function^[91]. Liver transplantation has recently been shown to revert cardiac alterations, including diastolic

dysfunction^[93]. It has been proposed that a diastolic dysfunction precedes systolic dysfunction in early heart disease and that antialdosterone treatment improves cardiac function. Pozzi *et al* recently demonstrated that antialdosterone treatment with K-canrenoate in cirrhosis ameliorated cardiac structure and function, but had almost no effects on systolic and diastolic functions^[98]. It is also possible that antialdosterone treatment may have beneficial effects on catecholamine-induced cardiac fibrosis, as described in heart failure^[99].

The clinical significance of diastolic dysfunction and the importance in cirrhotic cardiomyopathy have been questioned, as overt cardiac failure is not prominent in cirrhosis. However, there are several reports of unexpected death from heart failure following liver transplantation^[100], surgical portocaval shunts and TIPS^[81]. These procedures involve a rapid increase in cardiac preload. In a less compliant heart, the diastolic dysfunction could be enough to cause pulmonary edema and heart failure. This is consistent with the findings of Huonker *et al*^[82], who reported an increase in pulmonary artery pressure, pre-load, and diastolic dysfunction after TIPS. Diastolic dysfunction could thus account for part of the cardiac dysfunction in cirrhotic cardiomyopathy.

Electromechanical abnormalities

The sympathetic nervous activity influences the heart rate and electromechanical coupling by several mechanisms: noradrenaline binding to beta-receptors, receptor-mediated G protein interaction, and consequently stimulation of adenylcyclase, activation of cAMP-dependent phosphokinase A, and channel phosphorylation. Several receptor and post-receptor defects have been described in cirrhosis with reduced beta-receptor density and sensitivity^[101], and altered G protein and calcium channel functions^[102]. All these defects may explain both impaired chronotropic responses and electromechanical uncoupling. This coupling between the cardiac contractions and the arterial system is of major importance to the amount of work performed by the left ventricular myocardium, and thereby, of the strain on the heart^[85]. The ascending aorta and aortic arc are the most compliant systemic arteries in the body. The ability to contain the entire stroke volume without excessive deflection in the arterial systolic pressure profile is of crucial importance, especially in patients with a large CO and stroke volume. On the other hand, too compliant a central arterial system will be unable to perform a timely and prompt delivery of blood to the different parts of the body, but may delay the flow to important areas of the vascular bed. Thus, the heart and central arterial tree work together in an essential coordination of the oscillating blood flow. This is especially important when vascular beds with highly different hemodynamic resistances are connected to the central arterial system, as in chronic liver disease.

In addition to the abnormal function of the calcium channels, Ward *et al*^[103] have shown a decrease in K⁺ currents in ventricular cardiomyocytes from cirrhotic rats, which prolong the *Q-T* interval. A prolonged *Q-T* interval is often present in chronic liver disease, potentially leading to ventricular arrhythmias and sudden cardiac

death^[75,77,104]. Bernardi *et al* have reported a prolonged *Q-T* interval, which is significantly related to the severity of the liver disease, plasma noradrenaline, and survival^[105]. A reversal of the *Q-T* interval seems to occur with improved liver function, for instance, after orthotopic liver transplantation^[106]. Results from our group indicate that the frequency adjusted *Q-Tc* becomes partly normalized after oral β -blocker treatment^[107]. The prolonged *Q-T* interval in cirrhosis should be considered as an element in the cirrhotic cardiomyopathy and may be of potential use in identifying patients at risk^[77]. Pathophysiological and clinical research is needed to assess the prognostic and therapeutic significance of the prolonged *Q-Tc* interval in chronic liver disease.

It can be concluded that there is evidence of a cirrhotic cardiomyopathy in cirrhosis that appears to be unmasked by procedures that stress the heart, such as pharmacological vasoconstriction, exercise, and by such portosystemic shunt procedures as insertion of TIPS. Future studies should be directed against an operable definition of cirrhotic cardiomyopathy, a delineation of the clinical importance, and potential treatment regimens. Until then, we still do not know how or if cirrhotic cardiomyopathy should be treated specifically.

CARDIOVASCULAR REGULATION IN CIRRHOSIS

Autonomic dysfunction

Evidence of autonomic defects in patients with cirrhosis has emerged from hemodynamic responses to standard cardiovascular reflex tests, such as Valsalva ratio, heart rate variability, and isometric exercise^[87,108-111]. Most studies of these issues have found a high prevalence of autonomic dysfunction in cirrhosis with associations to liver dysfunction and survival^[111,112]. The results of Mohamed *et al* suggest that the autonomic dysfunction is temporary, arises as a consequence of liver dysfunction, and may be reversible after liver transplantation^[106]. Whereas most studies have focused on the defects in the SNS, recent papers have emphasized the importance of a vagal impairment for sodium and fluid retention^[108,110,112]. Sympathetic responses to dynamic exercise seem to be normal in patients with cirrhosis, but those to isometric exercise are clearly impaired^[113,114]. Similarly, blood pressure responses to orthostasis are impaired, probably because of a blunted baroreflex function^[87,115,116]. Abnormal cardiovascular responses to pharmacological stimulations with angiotensin II, noradrenaline, and vasopressin in terms of impaired responses in blood flow and blood pressure have also been reported in cirrhosis^[117-119]. Dillon *et al*^[112] have described the correction of autonomic dysfunction in cirrhosis by captopril, which indicates that vagal dysfunction in cirrhosis is partly caused by neuromodulation by angiotensin II. Involvement of the RAAS is also supported by the data from La Villa *et al*, who recently reported that canrenone, an aldosterone antagonist, normalized cardiac responses to postural changes in compensated cirrhotic patients^[116].

At present the pathophysiological basis of the autonomic dysfunction is unknown, but it could be within the central nervous system through damage to

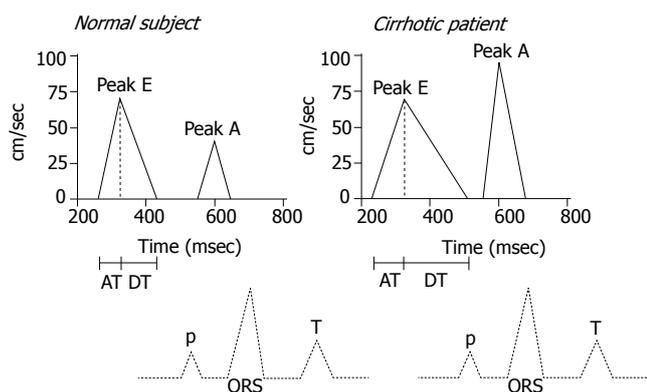


Figure 3 Diagram of the transmitral Doppler flow signal in the healthy state and in a patient with cirrhosis. The Doppler flow profile reflects the early filling of the ventricle (Peak E) and the late atrial contribution to its filling (Peak A). A decrease in the E/A ratio and prolonged acceleration time (AT) and deceleration time (DT) indicate diastolic dysfunction, as illustrated in the right panel. The broken lines are the time-related electrocardiogram.

the peripheral nerves or changes in neurotransmission in terms of a post-receptor defect which could explain the vascular hyporeactivity (Figure 4). From the amount of data available, a multifactorial etiology to the hyporesponsiveness in cirrhosis seems most likely.

Vascular hyporeactivity and arterial compliance in cirrhosis

The hyporeactivity of the vascular system in chronic liver disease is probably a result of a differential balance between vasoconstricting and vasodilating forces in different vascular areas (Figure 2). Generally, however, the vascular system in cirrhosis is very flexible as reflected by an overall increased vascular and arterial compliance^[120-122]. The systemic arterial compliance, defined as an increase in intra-arterial volume relative to an increase in transmural arterial blood pressure, is especially increased in patients with decompensated cirrhosis^[46]. This is because of the changes in the arterial wall, as well as dynamic changes, and is closely associated with the circulatory and homeostatic derangement^[47,122]. Therefore, the changes in arterial mechanics are partly reversible. The arteriolar tone adjusts the level of blood pressure and may thereby also affect large artery compliance. In fact, arterial compliance depends on the properties of arterial intrinsic elastic and smooth muscle, whereas arteriolar tone should result more from the balance between vasoconstrictors and vasodilators. The increased arterial compliance is directly related to the severity of liver disease and to the circulating vasodilator, CGRP, but is inversely related to circulating adrenaline, and unrelated to indicators of potent vasoconstrictor systems (SNS and ET-1)^[47,123]. In addition, other operative elements in the abnormal arterial compliance are blood volume abnormalities, hypoxia, and abnormalities in the C-type natriuretic peptide (CNP), but not to arterial natriuretic peptide. Arterial compliance is not affected by β -adrenergic blockade, but terlipressin almost normalizes it^[124]. Arterial compliance is an important determinant of the coupling between the heart and the arterial system, and of the dynamics

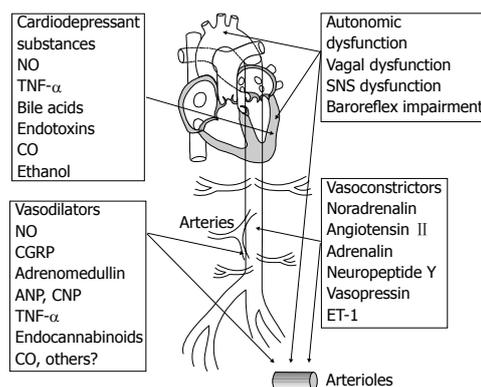


Figure 4 Vascular hyporeactivity in cirrhosis may originate in the central nervous system, the autonomic nervous system, from local mediators, or within the smooth muscle cell/heart muscle cell. An autonomic dysfunction may act at cardiac, arterial, and arteriolar levels. Vasodilators and vasoconstrictors may act variably at cardiac, arterial, and arteriolar levels. At the smooth cellular (arteriolar) level, hyporeactivity may be caused by increased concentrations of vasodilators (NO, nitric oxide; CGRP, calcitonin gene-related peptide; ANP, atrial natriuretic peptide; TNF- α , tumor necrosis factor-alpha; endocannabinoids; CO: carbon monoxide) and/or decreased sensitivity to vasoconstrictors (ET-1: endothelin-1).

of intravascular volume relocation^[125]. An element in the elevated arterial compliance in advanced cirrhosis is the reduced arterial blood volume and blood pressure^[47].

Recent data suggest that the hyperdynamic circulation is mainly caused by circulatory alterations in the splanchnic area^[67]. Thus, arteriolar vasodilatation is a more localized event, whereas the increased arterial compliance is more general^[47]. Arterial compliance may therefore be an integral variable for vascular responsiveness, together with the systemic vascular resistance. Changed dynamic and static function of the arterial tree may contribute to the abnormal reactions of volume and baroreceptors, and have implications for the abnormal circulatory regulation, and potentially for therapy with vasoactive drugs. These aspects are, however, a topic for further research. In conclusion, arterial compliance is elevated in advanced cirrhosis. Besides a relation to age, body size, gender, and the level of the arterial blood pressure, arterial compliance is directly related to the severity of cirrhosis and the hyperdynamic circulatory derangement.

Baroreceptor function and homeostasis of arterial blood pressure in cirrhosis

The arterial vasodilatation contributes to the displacement of the central blood volume towards peripheral and splanchnic vascular regions, resulting in central and arterial hypovolaemia and activated counter regulatory mechanisms^[65,71,126]. The pronounced vasodilatation and the fall in arterial blood pressure elicit reduced baroreflex activity and diminished central signaling from the cardioinhibitory center and results primarily in SNS-mediated vasoconstriction of resistance vessels^[127]. The normal response to upright posture is a fall in arterial, central venous, and pulse pressures and there is a close relation between the decline in pulse pressure on the one hand and the rising heart rate and declining splanchnic blood flow on the other, which suggests that arterial baroreceptors initiate an increase in heart rate and

splanchnic vasoconstriction^[127]. Whether this pattern is disturbed in cirrhosis is unclear at present. Some studies have shown that a reduction in thoracic blood volume increases the sensitivity of the arterial baroreflex^[127,128]. A resetting of the baroreceptors is still discussed in human conditions in relation to wall tension of the fibroelastic tissues in the vessels and stretch-induced activation of the sodium-potassium channels^[127]. As mentioned above, autonomic dysfunction is well-established in cirrhosis^[112,114,129], and impaired baroreceptor reflex sensitivity has been suggested^[121,115,130]. The baroreceptor function is influenced by hypoxia^[131,132] which is often seen in cirrhosis and in particular in those patients with the hepatopulmonary syndrome^[133,134].

Baroreceptor sensitivity in the small subset of cirrhotic patients with arterial hypertension has not been investigated, but it is very likely that the co-existence of two different conditions of baroreceptor function involved may affect the cardiovascular regulation^[135]. Future studies should seek to reveal the direction of this possible baroreceptor dysfunction in this specific group of patients.

Blood pressure regulation

Potent vasodilators such as NO, CGRP, histamine, bradykinin, and serotonin have been implicated in the regulation of the blood pressure in chronic liver disease^[1]. A significant inverse relation of the potent vasodilator, adrenomedullin, to arterial blood pressure and ET suggests that these two vasoactive systems play a role in blood pressure regulation in cirrhosis. NOS blockade causes higher arterial blood pressure in cirrhotic rats. Inhibition of the endocannabinoid CB1 receptor raises arterial blood pressure in experimental cirrhosis, and anandamide from the monocytes of cirrhotic rats may contribute to the arterial hypotension observed^[132,136]. However, the significance of blood pressure dysregulation in human cirrhosis awaits further studies.

The arterial blood pressure is kept low below normal, depending on the state of the disease, as a circulatory compromise between the vasodilating and counter regulatory vasoconstricting forces affecting both vascular resistance and compliance. The arterial blood pressure possesses a circadian variation. Twenty-four-hour determinations in cirrhotic patients show that during the day, the systolic, diastolic, and mean arterial blood pressures are substantially reduced, whereas at night, the values are unexpectedly normal^[4]. In cirrhosis, the drop from day time to night-time and the rise from night-time to daytime show lower values than in controls. It is known from several diseases, such as uraemia and different types of heart failure, that the circulation of patients classified as "nondippers" is abnormally regulated. The combination of normal blood pressure and increased heart rate at night suggests abnormal regulation of the circulation in cirrhosis. Prolonged rest in the supine position (as during sleep) may lessen the abnormal distribution of the blood volume and improve the ability to maintain a normal "sleeping" arterial blood pressure, only at the cost of an increased heart rate and CO. The upright position further aggravates central hypovolaemia, and normal arterial blood pressure cannot be maintained, even when the heart rate and CO

are increased^[20,21,137]. The negative correlation of the arterial blood pressure to the Child score during the day and at night confirms that the hemodynamic derangement is related to the severity of the liver disease^[69]. The low arterial blood pressure, the abnormal distribution of the circulating medium and diurnal variation in arterial blood pressure, and the marked activation of neurohumoral systems contribute to the abnormal homeostatic regulation in patients with cirrhosis.

Previous reports of findings that arterial hypertension does not occur together with cirrhosis of the liver are not true in an absolute sense. However, the prevalence of arterial hypertension in cirrhotic patients is substantially reduced, especially in advanced cirrhosis^[135]. As hypertensive patients are often effectively treated with diuretics, calcium channel antagonists, beta-blockers, ACE-inhibitors, etc., and some of these drugs are also applied in the treatment of cirrhosis and portal hypertension, the natural history and prevalence of cirrhosis in patients with arterial hypertension, arterial hypertension in patients with cirrhosis, and the interrelationship of these two diseases may be difficult to study today in prospective and untreated cases. Nevertheless, such studies are relevant, since there are many unsolved questions.

PULMONARY DYSFUNCTION IN CIRRHOSIS

Patients with cirrhosis often complain of dyspnea and platypnea, and arterial oxygenation is often impaired with orthodeoxia^[138,139]. The etiology of abnormal lung function and ventilation in cirrhosis may be multifarious and is often a combination of the presence of cardiac dysfunction, heavy smoking, and chronic obstructive lung disease which is common in patients with alcoholic cirrhosis^[140]. In addition, lung function and oxygenation can be affected by edema and tense ascites, which are ameliorated after diuretic treatment and paracentesis^[141]. But independent of smoking status, patients with cirrhosis have a compromised lung function with a reduced transfer factor and ventilation/perfusion abnormalities^[45,134,140,142] and arterial hypoxemia is seen in 30%-70% of patients with chronic liver disease, depending on the severity^[143,144]. Various pathophysiological factors may be involved in the reduced diffusing capacity, including an abnormal ventilation/perfusion ratio (V_A/Q), the presence of arterial venous shunts, and changes in the alveolar-arterial membrane (Figure 5).

Hepatopulmonary syndrome

The reduced diffusion capacity (transfer factor) has been related to the increased amount of blood in the lung capillaries^[145], but this does not seem to be the case, as there is a direct correlation between the amount of circulating red blood cells and flow in the lung capillaries and the diffusing capacity in normal physiology^[146]. To support this concept, we have earlier described direct relations between the diffusing capacity and the cardiac output and central blood volume in cirrhosis (Figure 5)^[45].

Pulmonary vascular resistance is most often decreased in cirrhosis^[147] and in a substantial number of patients there seem to be areas with a high perfusion rate in relation to alveolar ventilation^[45,140]. Besides the abnormal ventilation/

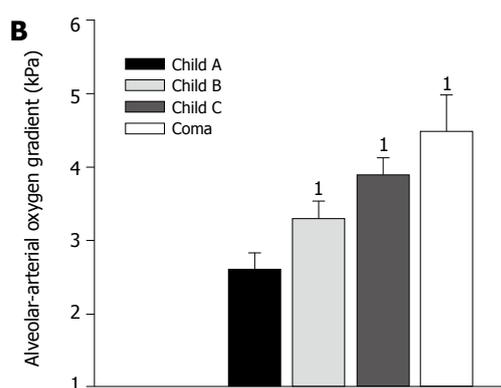
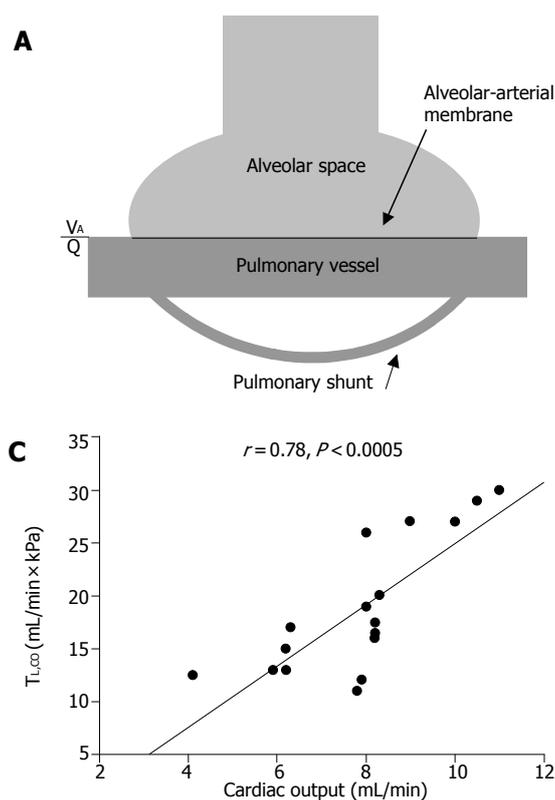


Figure 5 Panel A. Lung diffusion and blood oxygenation depend on the diffusion properties of the alveolar-arterial membrane, the degree of arterial-venous shunting through pulmonary shunts, and the degree of ventilation-perfusion inequality (V_A/Q). **Panel B.** The alveolar-arterial oxygen gradient increases with the severity of liver disease e.g. graduated according to the Child-Turcotte classification with the highest values in patients with hepatic coma. ¹denotes significant difference from Child class A patients (from Ref. [143]). **Panel C.** In cirrhosis, the diffusing capacity bears a direct relation to the increased cardiac output and to the central blood volume (not shown). Data from Ref. [45].

perfusion ratio and the presence of regular pulmonary arteriovenous shunts, intrapulmonary vascular dilatations have also been described^[134,140,147,148]. The condition with reduced transfer factor, abnormal ventilation/perfusion ratio or shunts, low arterial oxygen saturation, and pulmonary vasodilatation and hyperdynamics is termed as the hepatopulmonary syndrome^[134,149,150]. Pulmonary angiography of these patients has revealed two types of patterns with a spongiform appearance of the vessels and small arteriovenous communications^[134]. Fallon *et al.*^[151-153] have recently reported increased pulmonary vascular endothelial NOS and increased production of cholangiocyte endothelin-1 and increased expression of endothelin-B receptors. Experimental NO-dependent vasodilatation is supported by clinical studies showing increased NO in the exhaled air of cirrhotic patients^[154,155]. A recent paper has shown increased carboxyhemoglobin levels that correlate with arterial oxygen tension and alveolar-arterial oxygen gradient in patients with the hepatopulmonary syndrome^[156].

The frequency of the hepatopulmonary syndrome in patients with cirrhosis is not yet established. Different reports have given different frequencies of reduced arterial oxygen saturation in cirrhotic patients varying from about 10% to as high as 70%^[143,157,158].

The degree of gas exchange abnormalities, such as the oxygen tension and the alveolar-arterial oxygen gradient, correlates with the severity of liver disease (Figure 5). The diagnosis of the hepatopulmonary syndrome relies on the demonstration of arterial hypoxemia ($PaO_2 < 9.31$ kPa), an age-adjusted increased alveolar-arterial oxygen gradient (> 2.66 kPa) and intrapulmonary vasodilatation^[159,160]. According to the severity of deoxygenation, four stages have been proposed^[160]. A 100% oxygen shunt study with the

patient breathing 100% oxygen may discriminate between functional and anatomic shunts^[154]. Contrast-enhanced echocardiography is considered as the method of choice in the diagnosis of the hepatopulmonary syndrome^[161]. Agitated saline (microbubbles) is injected into a brachial vein and the bolus is shortly seen in the right heart chambers. A positive test for intrapulmonary vasodilatation occurs with delayed visualization of the microbubbles in the left heart chambers after more than three heart beats^[162]. Finally, a lung perfusion scan with the injection of macroaggregated albumin and estimation of the extra-pulmonary shunt fraction can be used^[148]. From counts over lungs and brain, the shunt fraction can be calculated and a value $> 6\%$ is considered positive with a sensitivity of 85%^[148,160].

No specific treatment, apart from long-term oxygen therapy, is available for the hepatopulmonary syndrome. It has been reversed by successful orthotopic liver transplantation in some patients^[144,163] and by insertion of a TIPS in others^[164]. TIPS insertion increases pulmonary artery pressure but cardiorespiratory complications are common and TIPS cannot be recommended for this indication at present^[165,166].

Portopulmonary hypertension

The association between portal hypertension and pulmonary artery hypertension is termed portopulmonary hypertension and is defined as a mean pulmonary artery pressure > 3.325 kPa and pulmonary vascular resistance > 120 dyn·s/cm⁵, and normal left atrial pressure (< 1.995 kPa)^[147]. It is seen infrequently in cirrhosis with an average prevalence from 1% to 4%^[167]. Symptoms are typically progressive and include fatigue, dyspnea, and edema^[134]. Systemic vascular resistance and cardiac output are not different from that of cirrhotic patients

without portopulmonary hypertension, whereas their arterial oxygenation is impaired^[168]. The histological appearance of pulmonary vessels is similar to that seen in primary pulmonary artery hypertension, and includes smooth muscle proliferation and hypertrophy^[160]. Local vasoconstrictor systems, like the endothelin system, may play a role and recently the administration of a mixed ET-antagonist has showed beneficial effects in portopulmonary hypertension^[169,170]. Treatment of portopulmonary hypertension is in general non-specific and palliative, and includes vasodilators, such as calcium channel blockers, nitrates, and prostacyclin^[160].

CONCLUSION

So far, a huge body of research has revealed that, in addition to portal and splanchnic complications to chronic liver disease, complications relating to the systemic and pulmonary circulation affect the prognosis of the patient as part of a multi-organ syndrome. Splanchnic vasodilatation in relation to portal hypertension is responsible for the hyperdynamic circulation and abnormal distribution of blood volume with a reduced "effective arterial blood volume" and activation of baroreceptor and volume-receptor reflexes as the outcome. The enhanced vasodilatation and counter regulatory over-activity of vasoconstrictor systems play major roles in the development of the multi-organ failure in cirrhosis with impaired function and perfusion of kidneys, lungs, brain, skin, and muscles. The function of the heart in cirrhosis is disturbed, with an increased cardiac output and heart rate. Left atrial and ventricular volumes tend to be slightly dilated, whereas the cardiac pressures are normal at rest. Cardiac performance and the systolic and diastolic functions are clearly impaired, in relation to the degree of liver dysfunction. The impaired cardiac contractility, termed cirrhotic cardiomyopathy, is different from that seen in alcoholic heart muscle disease. Reduced β -adrenergic receptor signal transduction and a defective cardiac excitation-contraction coupling are among the significant pathophysiological mechanisms. The cirrhotic heart is overloaded with a high-output failure and at the same time hyperdynamic and dysfunctional, and strain may unmask latent heart failure. In addition, the circulation and the function of a variety of organs are disturbed, including the lungs, kidneys, brain, and peripheral tissues. No specific treatment can be recommended and the only radical treatment option is liver transplantation. A considerable number of patients present with reduced pulmonary vascular resistance, impaired ventilation, and hypoxemia as part of a hepatopulmonary syndrome. A few patients develop portopulmonary hypertension with increased pulmonary vascular resistance.

Although there are still major unsolved questions that remain to be answered, the circulatory and neuroendocrine derangements play important roles in the clinical aggravation, hepatopulmonary dysfunction, and circulatory reactivity. This aspect is important to take into account in the clinical handling of the patient and the assessment of the prognosis.

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Role of platelet-activating factor in pathogenesis of acute pancreatitis

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Abstract

Platelet-activating factor (PAF) is a potent proinflammatory phospholipid mediator that belongs to a family of biologically active, structurally related alkyl phosphoglycerides with diverse pathological and physiological effects. This bioactive phospholipid mediates processes as diverse as wound healing, physiological inflammation, angiogenesis, apoptosis, reproduction and long-term potentiation. PAF acts by binding to a specific G protein-coupled receptor to activate multiple intracellular signaling pathways. Since most cells both synthesize and release PAF and express PAF receptors, PAF has potent biological actions in a broad range of cell types and tissues. Inappropriate activation of this signaling pathway is associated with many diseases in which inflammation is thought to be one of the underlying features. Acute pancreatitis (AP) is a common inflammatory disease. The onset of AP is pancreatic autodigestion mediated by abnormal activation of pancreatic enzyme caused by multiple agents, which subsequently induce pancreatic and systemic inflammatory reactions. A number of experimental pancreatitis and clinical trials indicate that PAF does play a critical role in the pathogenesis of AP. Administration of PAF receptor antagonist can significantly reduce local and systemic events that occur in AP. This review focuses on the aspects that are more relevant to the pathogenesis of AP.

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INTRODUCTION

Inflammatory reaction is a common pathophysiological process. Appropriate inflammatory reaction has protective effects, but excessive reaction often induces injury. The underlying pathological changes of inflammation include exudation, alteration, and proliferation, which are the outcomes mediated by inflammatory mediators such as platelet-activating factor (PAF), leukotriene, tumor necrosis factor alpha (TNF- α), and interleukin-1 (IL-1). Numerous researches have shown that PAF is one of the most potent mediators in many inflammatory processes, and not only induces inflammatory reaction but also mediates synthesis and release of other mediators to aggravate the degree of inflammation.

Acute pancreatitis (AP) is a common clinical inflammatory disease. A single injection of PAF into the superior pancreaticoduodenal artery of rabbits induces dose-dependent morphologic alterations of pancreatic tissue and increases serum amylase levels^[1]. Murine pancreatic acini synthesizes PAF^[2] and pancreatic vascular endothelium expresses PAF receptor^[3]. These findings suggest that this mediator may have a role in AP. AP is usually classified into mild and severe type. Severe acute pancreatitis (SAP) still has a high mortality rate, while the pathogenesis of AP is not well-defined. There is growing evidence that pathogenetic factors deciding the severity of AP are complicated. PAF has been strongly implicated in the development of AP.

Here we have reviewed the role of PAFs such as excessive leukocyte stimulation, microcirculatory disorder, gut endothelial barrier dysfunction, bacterial translocation, acinar cell necrosis and apoptosis in the pathogenesis of AP.

BIOLOGICAL CHARACTERISTICS OF PAF

Phospholipids are the major components of cellular membrane and are also known to be the source of arachidonic acid, which is metabolized into bioactive eicosanoids. Some phospholipids, including lysophosphatidic acid, sphingosine-1-phosphate, and PAF, exert bioactive effects. The term PAF is used because it

was first described as the substance responsible for the aggregation of platelets released from rabbit basophils after IgE stimulation^[4]. Although PAF is still commonly used, it has diverse and potent physiological effects^[5,6]. The chemical structure of PAF is 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine determined in 1979 by three independent laboratories^[7-9].

Two distinct pathways for the synthesis of PAF have been demonstrated: the remodeling pathway and *de novo* pathway^[10]. The remodeling pathway is mainly involved in the synthesis of PAF by stimulated inflammatory cells. The activities of lyso-PAF acetyltransferase (lyso-PAF AcT) and PAF-synthesizing phosphocholine transferase (PAF-PCT) are directly responsible for PAF synthesis. Lyso-PAF AcT catalyzes the transfer of acetyl moiety from acetyl CoA to free hydroxyl at sn-2 position of 1-alkyl-sn-glycero-3-phosphorylcholine. PAF-PCT catalyzes the conversion of 1-alkyl-2-acetyl-sn-glycerols to PAF^[11]. The synthesis and catabolism of PAF are highly regulated. The final molecular composition of PAF in tissues and the expression of its biological activities depend on the activation of catabolic pathways. The most important enzyme in limiting the PAF bioactivity is a PAF-specific acetylhydrolase (PAF-AH), which cleaves the short acyl chain at sn-2 position and forms biologically inactive lyso-PAF^[12].

A diverse array of cells has been shown to synthesize PAF upon appropriate stimulation. In particular, PAF is produced by a variety of cells such as monocytes/macrophages, polymorphonuclear leukocytes (PMN), eosinophils, basophils, platelets, mast cells, vascular endothelial cells, and lymphocytes, which may participate in the inflammatory reaction^[5,13]. Murine pancreatic acini can also synthesize PAF induced by cerulein^[2].

PAF is a phospholipid mediator possessing a wide spectrum of potent proinflammatory action. *In vitro*, PAF promotes chemotaxis, aggregation, granule secretion, and oxygen radical generation of leukocytes and adherence of leukocytes to the endothelium. Moreover, PAF is involved in allergy, wound healing, atherosclerosis, angiogenesis, apoptosis, reproduction, and long-term potentiation^[5,6,13]. Recent findings have revealed some novel effects of PAF. Through inflammatory cytokines, estrogen depletion enhances PAF production as a unique autocrine factor for osteoclast functions. Inhibition of PAF function might pave the way for a new strategy to prevent postmenopausal bone loss without disturbing osteoblast functions^[14]. The study investigating the functional PAF receptor on cell and nuclear surfaces of leukemic B cells in chronic lymphocytic leukemic patients showed that the potent immunoregulatory role of PAF in B cell physiology and the presence or absence of PAF receptor on leukemic B cells may profoundly affect their *in vivo* behavior^[15]. In jet fuel-induced immune suppression, PAF receptor binding can modulate immune function and is an early event in the induction of immune suppression by immunotoxic environmental agents targeting the skin^[16]. PAF can amplify the heterogeneity between ischemic and normal cardiac myocytes during ischemia/reperfusion which might play a vital role in the pathogenesis of arrhythmia induced by ischemia/reperfusion^[17], increase the expression of nerve

growth factor mRNA and protein in human astrocytes under hypoxia which may protect the nervous tissue by promoting neuronal survival^[18]. PAF is also involved in the etiopathogenesis of type 1 diabetes^[19] and the pathogenesis of acute liver failure as well as in augmented compensatory liver tissue repair post-acetaminophen treatment^[20].

PAF acts by binding to a specific receptor, subsequently activates multiple intracellular signaling pathways in various cell types. PAF receptor belongs to G protein-coupled receptor subfamily^[21,22]. Most cells that produce PAF possess PAF receptors and are targets for PAF action. The pancreatic vascular endothelium also expresses PAF receptor^[3]. It has been demonstrated that PAF binds to the receptor and activates the associated G protein. In turn, G protein activates a phosphatidylinositol-specific PLC which hydrolyzes a membrane phospholipid, phosphatidylinositol 4, 5-bisphosphate (PIP₂), to generate two second messengers: diacylglycerol and inositol 1,4,5-trisphosphate (IP₃). These compounds mediate the release of Ca²⁺ from intracellular store (ER) and activation of protein kinase C (PKC), respectively. Moreover, it has been shown that PAF can activate mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase (ERK)^[23-27], p38 MAPK^[25-27], and c-Jun N-terminal kinase (JNK)^[28]. Using human umbilical vein endothelial cells (HUVECs) as a model system, Deo *et al.*^[29] suggested for the first time that PAF activates pertussis toxin-insensitive G_{αq} protein upon binding to its seven transmembrane receptors and adenylate cyclase, as well as elevates cAMP levels, which activate protein kinase A (PKA). PAF exposure induces the expression of TIMP2 and MT1-MMP and binding of p³⁰Cas, Src, SHC, and paxillin to FAK. It was found that PAF is capable of stimulating nuclear factor kappa B (NF- κ B) activation and transcription of c-fos and c-jun genes in inflammatory cells^[30,31].

INVOLVEMENT OF PAF IN THE PATHOGENESIS OF AP

AP is a common clinical condition and is usually classified into mild and severe type. SAP is characterized by acute morbidity, rapid progression, multiple complications, and high mortality rate. While the pathogenesis of AP is not well-defined, considerable advances have been made in this research field. At present, it is considered that severity of pancreatitis is associated with excessive leukocyte stimulation, microcirculatory disorder, gut endothelial barrier dysfunction, bacterial translocation, and acinar cell necrosis and apoptosis. In the pathogenesis of AP, PAF has been strongly implicated (Table 1).

Role of PAF in excessive leukocyte stimulation

In the course of AP, abnormal intra-acinar cell activation of digestive enzymes induced by diverse stimuli triggers morphopathological changes in pancreatic and adjacent tissues, such as inflammation, edema, hemorrhage, necrosis, and even systemic manifestations. In 1988, Rinderknecht^[32] proposed the hypothesis of excessive leukocyte stimulation. Consequently, a great number of studies have demonstrated that the initial injury results in the expression of inflammatory mediators such as PAF.

Table 1 Important roles of PAF in pathogenesis of AP

| Main pathogenesis of AP | Important roles of PAF |
|-------------------------------------|--|
| Excessive leukocyte stimulation | To facilitate adhesion, chemotaxis and degranulation and to mediate the production of inflammatory mediators of leukocytes, and to delay PMN apoptosis |
| Microcirculatory disorder | To modulate indirectly/directly microvascular permeability |
| Gut endothelial barrier dysfunction | To upregulate adhesion molecules, intercellular signals and leukocyte-endothelial cell interactions |
| Bacterial translocation | To depend on its ability to induce gut endothelial barrier dysfunction |
| Acinar cell necrosis and apoptosis | To mediate neutrophil chemotaxis and delay neutrophil apoptosis in the pancreas, but neutrophils convert acinar cells undergoing apoptosis into necrotic cells |

These mediators mediate activation and infiltration of leukocytes, a subsequent systemic inflammatory response, and multiple organ injury.

In vivo and *in vitro* studies suggest that proteolytic enzyme trypsin stimulates cytokine production from macrophages^[33]. In an idealized *in vitro* experiment mimicking cerulein-induced AP, cerulein induces amylase release, increases $[Ca^{2+}]_i$ and PAF synthesis of cultured pancreatic acini^[2]. PAF plays a role in inflammatory reaction and induces expression of adhesion molecules that mediate neutrophil accumulation. However, PAF antagonists reduce expression of adhesion molecules and the severity of inflammation when given immediately after the induction of mild AP in mice^[34]. Another research showed that PAF mediates macrophages to release thermolabile neutrophil chemotactic protein that induces neutrophil migration^[35]. Moreover, activation of neutrophils with zymosan leads to the activation of PAF receptors followed by activation of CD11/CD18, phagocytosis of zymosan particles and subsequent IL-8 release. The production of IL-8 by neutrophils in response to particulate stimuli may play a role in the recruitment and activation of neutrophils in inflammatory reaction^[36]. In addition, PAF is able to enhance superoxide production of PMN, expression of CD11b, and release of elastase, which are essential factors in the pathophysiology of multiple-organ failure^[37]. PAF can delay apoptosis of PMN and amplify the inflammatory response by activating ERK signaling pathway^[38,39]. Furthermore, PAF induces NF- κ B activation and regulates gene expression through G protein-coupled transcription factor activation pathway^[40]. NF- κ B is a key element in inflammatory responses based on its ability to regulate the expression of inflammatory mediators. Activation of NF- κ B within the pancreas is sufficient to initiate an inflammatory response^[41].

The above findings indicate that PAF is involved in adhesion, chemotaxis, degranulation and, the whole procedure of activation of leukocytes. Besides, it can amplify inflammatory response via delaying apoptosis of PMN. PAF receptor is almost ubiquitous in diverse type cells and acts not only on local pancreas but also on distant organs to induce systemic inflammatory response and multiple organ injury.

Role of PAF in microcirculatory disorders

A number of experimental studies suggest that pathogenesis of AP correlates with microcirculatory disorders. Many complications of SAP are due to the amplifying effects of microcirculatory disruption. Constriction of interlobular pancreatic arteries 2 min after intraductal infusion of sodium taurocholate has been observed, indicating that microcirculatory changes may occur in early AP^[42]. There is evidence that pancreatic microcirculatory changes are closely related to the process of AP. Ligating duodenum over half its circumference at 2 cm on either side of the duodenal entry of the biliopancreatic duct induces histopathologic alterations of the pancreas such as edema, parenchymal necrosis, thrombosis and hemorrhage, indicating that tissue ischemia plays a role in increasing the severity of pancreatitis^[43].

Many vasoactive mediators activated during the inflammatory response to pancreatic injury can cause microcirculatory disorders in AP. PAF is one of the most important mediator. Increased microvessel permeability caused by PAF may be related to direct endothelial cell activation, adhesion molecule expression, and leukocyte activation. Synthesis of PAF by endothelial cells at the site of plasmin generation may render the endothelial cell surface pro-adhesive for neutrophils and favor a local increase in vascular permeability^[44]. Recent data suggest that PAF is able to directly modulate microvascular permeability and increase venular permeability^[45]. Increased capillary permeability permits sequestration of macromolecules and fluid, which causes a deficiency of circulating blood volume and microcirculatory disorders. Predominant microcirculatory disorders are nutritive capillary perfusion failure, with the consequence of prolonged focal hypoxia or anoxia, and inflammation-associated microvascular leukocyte recruitment, CD11b and intercellular adhesion molecule (ICAM)-1-mediated leukocyte-endothelial cell interaction and loss of endothelial integrity, which may result in both edema formation and necrosis. Moreover, vasospasm and microthrombi formation due to hypercoagulability can also lead to the deterioration of pancreatic microcirculation and pancreatic necrosis.

It has been shown that the treatment of AP with PAF antagonists can significantly improve capillary blood flow in the pancreas and colon, renal, and respiratory function as well as survival rate, stabilize capillary permeability, decrease fluid loss into the third space^[46,47]. The partial protective effect of PAF antagonists further supports the role of PAF in microcirculatory disorders.

Role of PAF in gut endothelial barrier dysfunction

Gut endothelial barrier dysfunction is a critical factor for the development of tissue injury and organ dysfunction in AP. The study on dogs colonized with a strain of *Escherichia coli* (*E. coli* 6938K) bearing plasmid pUC4K showed that most dogs with severe pancreatitis have ischemic changes in the small bowel mucosa and *E. coli* translocation to the pancreas and mesenteric lymph nodes (MLNs), suggesting that the gut is a primary source of infection in pancreatitis^[48].

PAF plays a key role in the development of pancreatitis-associated gut endothelial barrier dysfunction and acts via the upregulation of adhesion molecules, intercellular signals and leukocyte-endothelial cell interactions. Leukocyte β 2-integrins play an important role in PAF-induced intestinal necrosis, and CD11b/CD18 are the main adhesion molecules involved in the pathogenesis of injury^[49]. Moreover, PAF can activate rapidly intestinal xanthine oxidase (XO) by converting proteolytic xanthine dehydrogenase (XD) to XO in the ileal epithelium. This effect is mediated by neutrophils. XO is an important source of reactive oxygen species in the small intestine and its activation promotes PAF-induced PMN sequestration in the intestine, thus causing gut endothelial injury^[50]. PAF can alter the cytoskeletal structure of intestinal epithelium and cause the influx of FD-4 (an index of intestinal permeability) from intestinal lumen to systemic circulation, and induce tyrosine phosphorylation of E-cadherin and cadherin-associated proteins. These findings reveal the possible mechanism of PAF in modulating intestinal mucosal permeability, PAF modulates macromolecular movement across the intestinal mucosal barrier probably via tyrosine phosphorylation of E-cadherin and cytoskeletal alteration of enterocytes^[51]. In addition, gut microcirculatory disorder plays a pivotal role in endothelial barrier dysfunction.

Further support for the role of PAF in gut endothelial barrier dysfunction is provided by the fact that the administration of PAF antagonists improves pancreatitis-associated gut barrier dysfunction characterized by increased endothelial permeability, albumin leakage from blood to the mucosal interstitium and intestinal lumen, and bacterial translocation^[52-54].

Role of PAF in bacterial translocation

Bacterial translocation is an important source of pancreas infection in AP and is responsible for the high incidence of pancreas and distant infections occurring after AP. Support for the role of PAF in bacterial translocation is provided by the fact that PAF antagonists reduce bacterial translocation. Pretreatment with PAF antagonists, WEB-2170, lexipafant and BN52021, reduces bacterial translocation to distant organs other than the pancreas^[55]. Similar findings showed that AP induced by supramaximal cerulein stimulation significantly increases bacterial translocation in MLNs, pancreas, liver, spleen and blood^[56]. Both recombinant PAF-acetylhydrolase (rPAF-AH) and PAF receptor antagonist, BN52021, can decrease bacterial translocation in the pancreas and blood. In addition, rPAF-AH decreases bacterial translocation in MLNs. The action of PAF involved in bacterial translocation may be due to its ability to induce gut endothelial barrier dysfunction in AP.

Role of PAF in acinar cell necrosis and apoptosis

Pancreatitis is characterized by inflammation and death of acinar cells. Death can occur due to either necrosis or apoptosis. Kaiser *et al.*^[57] have observed marked necrosis but very little apoptosis in severe pancreatitis models. In contrast to the findings in severe pancreatitis, mild

pancreatitis is characterized by very little necrosis but a high degree of apoptosis, suggesting that apoptosis may be a teleologically beneficial response to acinar cell injury in general, especially in AP. Deficiency of pancreatic connexin converts reversible AP into severe disease and decreases the sensitivity of acinar cells to apoptotic stimuli, demonstrating that apoptosis determines the severity of AP^[58].

It has been reported that PAF is involved in acinar cell damage^[59]. Treatment with antineutrophil serum (ANS) and BN52021 can prevent inflammatory responses caused by cerulein and decreases cell damage. Treatment with ANS increases apoptosis in cerulein-infused animals, indicating that cerulein stimulates pancreatic production of PAF. PAF mediates both apoptosis and neutrophil chemotaxis in the pancreas. Neutrophils in turn may convert acinar cells undergoing apoptosis to necrotic cells.

THERAPEUTIC EFFECTS OF PAF ANTAGONISTS ON AP

Recent studies have established the critical role of inflammatory mediators such as TNF- α , IL-1 β , IL-6, IL-8, IL-10, PAF, C5a, ICAM-1 and substance P, in the progression of AP from local pancreatic inflammation to a systemic inflammatory disease. Elucidation of the key mediators in AP coupled with the discovery of specific inhibitors makes it possible to develop clinically effective anti-inflammatory therapy. At present, a number of inflammatory mediator antagonists are tested. PAF antagonists, including ginkgolide B (BN52021), lexipafant (BB-882), CV-6209, TCV309, and WEB-2170, *etc.*, have shown beneficial effects on the manifestations of AP. In experimental pancreatitis and clinical trials, administration of several PAF antagonists significantly reduces the level of serum amylase, leukocyte infiltration, and improves capillary blood flow in the pancreas and distant organs, renal and respiratory function, and survival rate.

In animal pancreatitis model, ginkgolide B could significantly reduce vascular permeability, pancreatic edema, hyperamylasemia, diminute superoxide dismutase (SOD) activity, and inhibit lipid peroxidation in pancreatic tissue. These changes are accompanied with significant reduction of acinar cell vacuolization and remarkable inhibition of inflammatory cell infiltration in the interacinar space^[60,61]. In addition, treatment with ginkgolide B has shown protective effects on slow mesenterio-angial small arteriolar and venular blood flow velocity and dilated mesenterio-angial small venular diameter at the early phase of AP^[62]. Moreover, ginkgolide B reduces bacterial translocation to distant sites, has a significant effect on serum pancreatic enzymes and histologic score of pancreatitis, and suppresses elevation in IL-6 levels. Preventing bacterial dissemination in early AP may have beneficial effects on the evolution of this disease^[55,56]. Furthermore, ginkgolide B reduces malondialdehyde accumulation in pancreatic tissue, prevents sulfhydryl depletion in lung tissue, necrotic and inflammatory changes in the pancreatic tissue, and improves survival rate^[63]. Activation of pulmonary alveolar macrophages (PAMs) might play an important role in

severe complications of AP. Ginkgolide B reduces total and free activity of lysosomal hydrolases of PAMs and partly prevents labilization of their lysosomal membranes. Therefore, an important mechanism of ginkgolide B underlying pulmonary complications of AP is to stabilize PAM lysosomes^[64]. Moreover, BN50739 can reduce intestinal injury, levels of endotoxin and bacterial counts in the portal blood, MLNs and pancreas and increase intestinal mucosal blood flow^[54]. In pigs with SAP, pre- and post-treatment with BN50739 can effectively reduce PAF levels in lung and tracheal mucosa and the severity of acute lung injury following SAP by reducing PMN sequestration and the amount of elastase, or by inhibiting PLA₂ activities in lung and tracheal mucosa^[65].

In rats with AP induced by intraductal infusion of 5 g/L sodium taurodeoxycholate, pretreatment with lexipafant could reduce pancreatic endothelial barrier dysfunction and severity of pancreatitis-associated intestinal dysfunction as well as systemic concentrations of IL-1 and local leukocyte recruitment^[53,66,67]. AP lexipafant reduces the activity of serum cytokines (TNF- α , IL-1 β), lung myeloperoxidase (MPO) and serum amylase^[68]. Lexipafant treatment can decrease bacterial spread to distant sites in AP induced by pressure injection of 3% taurocholate and trypsin into the common biliopancreatic duct^[69].

In rats, TCV-309 administered prior to cerulein and/or PAF reduces cerulein-induced pancreatitis and prevents PAF-induced pancreatitis^[70]. It was reported that treatment with TCV-309 before septic challenge effectively prevents hyperactivity of bronchoalveolar macrophages and pancreatitis-associated lung injury by reducing serum concentrations of cytokine-induced neutrophil chemoattractant (CINC) and CINC messenger RNA (mRNA) in the lung, as well as pulmonary infiltrates immunoreactive for CINC or Mac-1 (CD11b/CD18)^[71].

CONCLUSION

In the pathogenesis of AP, PAF exhibits pleiotropic function and is involved in both local pancreatic injury and systemic multiple organ damage. The effectiveness of PAF antagonists depends not only on their ability to block the effects of inflammatory mediators but also on their administration early enough in the course of pancreatitis before pancreatic necrosis or organ dysfunction occur. PAF antagonist therapy for systemic inflammatory response syndrome and multi-organ dysfunction syndrome in the management of patients with SAP has been considered as an important advance in the treatment of these patients.

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LIVER CANCER

Long-term outcomes of hepatectomy vs percutaneous ablation for treatment of hepatocellular carcinoma ≤ 4 cm

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are good candidates for hepatectomy, provided that the hepatic functional reserve of the patient permits resection.

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Key words: Liver neoplasms; Hepatocellular carcinoma; Hepatectomy; Percutaneous ablation; Prognosis; Multivariate analysis

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Abstract

AIM: To determine which treatment modality - hepatectomy or percutaneous ablation - is more beneficial for patients with small hepatocellular carcinoma (HCC) (≤ 4 cm) in terms of long-term outcomes.

METHODS: A retrospective analysis of 149 patients with HCC ≤ 4 cm was conducted. Eighty-five patients underwent partial hepatectomy (anatomic in 47 and non-anatomic in 38) and 64 underwent percutaneous ablation (percutaneous ethanol injection in 37, radiofrequency ablation in 21, and microwave coagulation in 6). The median follow-up period was 69 mo.

RESULTS: Hepatectomy was associated with larger tumor size ($P < 0.001$), whereas percutaneous ablation was significantly associated with impaired hepatic functional reserve. Local recurrence was less frequent following hepatectomy ($P < 0.0001$). Survival was better following hepatectomy (median survival time: 122 mo) than following percutaneous ablation (median survival time: 66 mo; $P = 0.0123$). When tumor size was divided into ≤ 2 cm vs > 2 cm, the favorable effects of hepatectomy on long-term survival was seen only in patients with tumors > 2 cm ($P = 0.0001$). The Cox proportional hazards regression model revealed that hepatectomy ($P = 0.006$) and tumors ≤ 2 cm ($P = 0.017$) were independently associated with better survival.

CONCLUSION: Hepatectomy provides both better local control and better long-term survival for patients with HCC ≤ 4 cm compared with percutaneous ablation. Of the patients with HCC ≤ 4 cm, those with tumors > 2 cm

INTRODUCTION

Hepatectomy and percutaneous ablation are the treatments of choice for small hepatocellular carcinoma (HCC)^[1,2]. Hepatectomy is recommended by surgeons, as this procedure removes portal venous thrombi in the adjacent liver^[3], allows for better local control^[4], and has better survival outcomes compared with percutaneous ablation^[5,6]. In contrast, gastroenterologists and interventional radiologists advocate percutaneous ablation techniques including ethanol injection^[7-9], microwave coagulation^[10-12], and radiofrequency ablation^[13-15], as these methods are not as invasive as hepatectomy^[7-15], have a lower incidence of morbidity and mortality^[7-13,15], are more feasible in patients with impaired hepatic functional reserve^[7-15], and have similar survival outcomes compared with hepatectomy^[7-9,15].

The aim of this study was to determine which treatment modality - hepatectomy or percutaneous ablation - is more beneficial for patients with small (≤ 4 cm) HCC in terms of long-term outcomes.

MATERIALS AND METHODS

Patients

From January 1990 to December 2002, 224 consecutive patients with HCC underwent either partial hepatectomy or percutaneous ablation therapy as an initial treatment at the Niigata University Medical and Dental Hospital. Of

these patients, 149 with HCC measuring ≤ 4 cm formed the basis of this retrospective study; they included 104 men and 45 women with a median age of 64 years (range: 29–83 years). Only HCCs measuring ≤ 4 cm were included in the current study, because percutaneous ablation therapy was the treatment modality mainly recommended for such tumors in our hospital. All patients were Japanese.

Treatment modalities

Of the 149 patients, 85 underwent a hepatectomy for HCC in the Division of Digestive and General Surgery. Hepatectomy. Procedures included non-anatomic hepatectomy in 38 patients, monosegmentectomy in 14, bisegmentectomy in 15, right hepatectomy in 14, and left hepatectomy in 4. In our department, the type of hepatectomy procedure selected was primarily based on the disappearance rate of indocyanine green (K_{ICG})^[16,17], which is an indicator of hepatic functional reserve. The selection criteria were $K_{ICG} \geq 0.12$ for hemihepatectomy, $K_{ICG} \geq 0.10$ for bisegmentectomy, $K_{ICG} \geq 0.08$ for monosegmentectomy, and $K_{ICG} \geq 0.06$ for non-anatomic hepatectomy (including the enucleation of hepatic tumors).

The remaining 64 patients underwent a percutaneous ablation procedure for HCC in the Division of Gastroenterology and Hepatology. Hepatectomy was considered as a feasible option for 51 of these 64 patients and was offered as an alternative. However, all these patients preferred to undergo percutaneous ablation treatment. Hepatectomy was not considered feasible in the remaining 13 patients due to impaired hepatic functional reserve ($K_{ICG} < 0.06$); therefore, these patients were only offered percutaneous ablation treatment. Percutaneous ablation procedures included percutaneous ethanol injection (PEI) in 37 patients, radiofrequency ablation (RFA) in 21, and microwave coagulation therapy (MCT) in 6. In these patients, adequacy of the ablation was assured with dual-phase dynamic computed tomography (section thickness of 5 mm) within a month of the procedure. A microwave tissue coagulator (Microtaze[®] OT-110M; Alfresa-Pharma Co., Inc., Osaka, Japan) and an RF generator (Cool-tip[®] RF System, CMI Century Medical Co., Inc., Tokyo, Japan) were introduced in our hospital in 1995 and 2000, respectively.

Post-treatment follow-up

There were no mortalities 30 d post-treatment in the current study. Serum concentrations of alpha-fetoprotein were measured and abdominal ultrasonography and/or contrast-enhanced computed tomography was performed on all the patients approximately 1 mo after the treatment. Thereafter, patients were regularly monitored for recurrences in outpatient clinics every 3 mo by physical examination, laboratory tests, and imaging studies. When intrahepatic recurrences were detected, they were treated with either interventional radiological techniques, such as PEI, RFA, MCT, transarterial chemoembolization and hepatic arterial infusion, or repeat hepatectomy when indicated. Patients with disseminated recurrences and those in a debilitated state were treated with supportive care.

The follow-up period after the treatment was defined as the interval between the date of the initial treatment

and that of the last follow-up, and ranged from 11 to 178 (median: 69) mo in the current study. The median follow-up period was 73 mo in patients who had undergone hepatectomy, and 61 mo in those who had undergone percutaneous ablation.

Definition of local recurrence after treatment

Local recurrence was defined as recurrences contiguous to resection margins in patients who had undergone hepatectomy, whereas in patients treated with percutaneous ablation, local recurrence was defined as recurrences contiguous to or within the ablated areas.

Laboratory examination

The following laboratory tests were performed before treatment: hepatitis B surface antigen; hepatitis C antibody; serum aspartate aminotransferase; serum alanine aminotransferase; the indocyanine green clearance test; and serum alpha-fetoprotein. Hepatitis B surface antigen and hepatitis C antibody in serum were detected by radioimmunoassay (Lumipulse II HBsAg; Fujirebio Co., Inc., Tokyo, Japan) and a second-generation enzyme-linked immunosorbent assay (Lumipulse II Ortho HCV; Ortho-Clinical Diagnostics Co., Inc., Tokyo, Japan), respectively. Indocyanine green (Diagnogreen; Daiichi Pharmaceutical Co., Inc., Tokyo, Japan) retention rate at 15 min after the injection of the dye (0.5 mg/kg) was used as an indicator of hepatic functional reserve, with a reference range of 10% or less^[16–18]. Serum concentrations of alpha-fetoprotein were determined by enzyme immunoassay (Luminomaster AFP; Sankyo Yell Yakuhin Co., Ltd., Tokyo, Japan).

Pathologic examination

Resected specimens from all the patients who had undergone hepatectomy were submitted to the Department of Surgical Pathology in our hospital. Each specimen was examined to determine the number of hepatic tumors, tumor size, vascular invasion (gross or microscopic), and cirrhosis. Vascular invasion included both portal and hepatic venous invasion in the current study. Cirrhosis in the adjacent (non-tumorous) liver was diagnosed microscopically based on the presence of regenerative nodules surrounded by fibrous septa. The pathology of the liver was confirmed by fine-needle biopsy in all the patients undergoing percutaneous ablation. Among this group of patients, 41 patients had a fine-needle biopsy of the tumor.

Factors influencing outcomes after treatment

To determine the factors that may influence outcomes after the treatment, 13 conventional variables^[19–22] were identified for univariate and multivariate analyses (Table 1). Vascular invasion and histologic grade were not chosen because they were often missed in patients undergoing percutaneous ablation.

Statistical analyses

Medical records and survival data were obtained for all the patients. The causes of death were determined from the medical records. Deaths from other causes were treated as uncensored cases. The Kaplan-Meier method was

used to estimate the cumulative incidences of events, and differences in these incidences were evaluated using the log rank test. The Cox proportional hazards regression model was performed to identify the factors that were independently associated with local recurrence and survival. In this model, a stepwise selection was used for variable selection with entry and removal limits of $P < 0.1$ and $P > 0.15$, respectively. The stability of each model was confirmed using a step-backward and step-forward fitting procedure, and variables identified as having an independent influence on local recurrence and survival were identical in both the procedures. Clinical features and pathologic tumor-related factors were compared between the two groups using Fisher's exact test. All statistical evaluations were performed using the SPSS 11.5J software package (SPSS Japan Inc., Tokyo, Japan). All tests were two-sided and P values of < 0.05 were considered statistically significant.

RESULTS

Clinicopathologic characteristics according to treatment modality

A total of 125 hepatic tumors (median: one per patient; range: 1-14 tumors) were resected in patients undergoing hepatectomy, and a total of 100 tumors (median: one per patient; range: 1-5 tumors) were treated in patients undergoing percutaneous ablation.

Patients treated with hepatectomy had larger tumors than those treated with percutaneous ablation. Patients who had undergone percutaneous ablation were characterized by hepatitis C virus infection, cirrhosis, Child-Pugh classification B or C, an impaired indocyanine green retention rate at 15 min, and increased serum concentrations of aspartate aminotransferase and alanine aminotransferase, suggesting impaired hepatic functional reserve with active hepatitis in these patients (Table 1).

Factors influencing local recurrence

During the follow-up period, local recurrences developed in two patients who had undergone hepatectomy and 17 patients who had been treated with percutaneous ablation (10 treated with PEI and 7 treated with RFA). Univariate analysis revealed that treatment modality ($P < 0.0001$), indocyanine green retention rate at 15 min ($P = 0.0005$), Child-Pugh classification ($P = 0.0012$), serum alpha-fetoprotein level ($P = 0.0072$), hepatitis C virus infection ($P = 0.0374$), and number of hepatic tumors ($P = 0.0419$) were risk factors for local recurrence. Of these six variables, multivariate analyses revealed that treatment modality and serum alpha-fetoprotein level were the only independently significant variables (Table 2).

Factors influencing long-term survival

At the time of disease status assessment, 53 patients who had undergone hepatectomy were alive, and 32 had died. Thirty-four patients treated with percutaneous ablation were alive, and 30 had died. Univariate analysis revealed that Child-Pugh classification ($P = 0.0010$), serum alpha-fetoprotein level ($P = 0.0052$), treatment modality ($P = 0.0123$), serum aspartate aminotransferase ($P = 0.0278$), tumor size

Table 1 Clinicopathologic characteristics of 149 patients with hepatocellular carcinoma according to treatment modality

| Variable | Hepatectomy | Percutaneous ablation |
|---------------------------|-------------|-----------------------|
| Age (yr) | | |
| ≤ 65 | 56 | 33 |
| > 65 | 29 | 31 |
| Gender | | |
| Male | 61 | 43 |
| Female | 24 | 21 |
| HBsAg | | |
| Negative | 61 | 54 |
| Positive | 24 | 10 |
| HCVAb | | |
| Negative | 38 | 11 |
| Positive | 47 | 53 ^b |
| Cirrhosis | | |
| Absent | 38 | 13 |
| Present | 47 | 51 ^b |
| Child-Pugh classification | | |
| A | 74 | 30 |
| B plus C | 11 | 34 ^b |
| ICG R15 (%) | | |
| ≤ 10 | 52 | 10 |
| > 10 | 33 | 54 ^b |
| AST(IU/L) | | |
| ≤ 50 | 48 | 17 |
| > 50 | 37 | 47 ^b |
| ALT(IU/L) | | |
| ≤ 50 | 50 | 23 |
| > 50 | 35 | 41 ^b |
| AFP (ng/mL) | | |
| ≤ 20 | 39 | 27 |
| > 20 | 46 | 37 |
| No. of hepatic tumors | | |
| Solitary | 67 | 41 |
| Multiple | 18 | 23 |
| Tumor size (cm) | | |
| ≤ 2 | 23 | 43 |
| > 2 | 62 | 21 ^b |

ICG R15: indocyanine green retention rate at 15 min. ^b $P < 0.01$ between groups.

Table 2 Independent risk factors for local recurrence

| Variable | Relative risk | 95% CI | P value |
|-----------------------|---------------|--------------|---------|
| Treatment modality | | | 0.001 |
| Hepatectomy | 1.000 | | |
| Percutaneous ablation | 13.442 | 3.102-58.254 | |
| AFP (ng/mL) | | | 0.014 |
| ≤ 20 | 1.000 | | |
| > 20 | 4.711 | 1.370-16.195 | |

($P = 0.0454$), and number of hepatic tumors ($P = 0.0464$) were significant prognostic factors of long-term survival. Of these six variables, multivariate analyses revealed that treatment modality and tumor size were the only independent significant variables (Table 3).

Outcomes after treatment according to tumor size

Local recurrence was significantly less frequent in patients who had undergone hepatectomy than in those who had undergone percutaneous ablation ($P < 0.0001$) (Figure 1). When the patients were divided into two groups according

Table 3 Independent factors influencing long-term survival

| Variable | Relative risk | 95% CI | P value |
|---------------------------|---------------|-------------|---------|
| Treatment modality | | | 0.006 |
| Hepatectomy | 1.000 | | |
| Percutaneous ablation | 2.398 | 1.278-4.499 | |
| Tumor size (cm) | | | 0.017 |
| ≤ 2 | 1.000 | | |
| > 2 | 2.159 | 1.148-4.060 | |
| Child-Pugh classification | | | 0.050 |
| A | 1.000 | | |
| B + C | 1.773 | 1.000-3.142 | |
| AFP (ng/mL) | | | 0.072 |
| ≤ 20 | 1.000 | | |
| > 20 | 1.713 | 0.952-3.084 | |

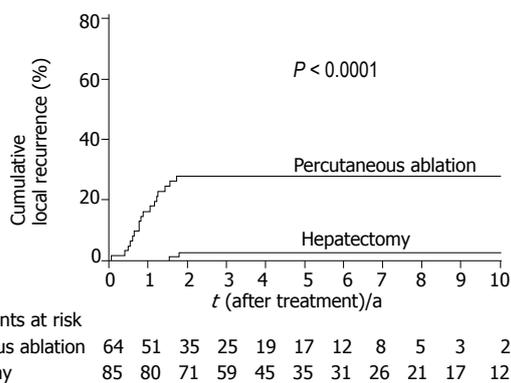


Figure 1 Kaplan-Meier estimates of local recurrence. The incidence of local recurrence reached a plateau of 28% at 20 mo after percutaneous ablation, and a plateau of 3% at 22 mo after hepatectomy.

to tumor size (≤ 2 cm vs > 2 cm), hepatectomy was found to be the more effective treatment for controlling local recurrence, but only in patients with HCC > 2 cm ($P < 0.0001$) (Figure 2). Survival after the treatment was significantly better in patients who had undergone hepatectomy than in those treated with percutaneous ablation ($P = 0.0123$, Figure 3). Again, hepatectomy was the more effective treatment in terms of long-term survival, but only in patients with HCC > 2 cm ($P = 0.0001$, Figure 4).

Incidence of vascular invasion according to tumor size

Vascular invasion was more frequent in patients with tumors > 2 cm (16/62, 26%) than in those with tumors ≤ 2 cm (1/23, 4%; $P = 0.033$, Table 4).

DISCUSSION

Selecting the correct treatment modality to suit individual patients with HCC remains a matter of debate^[1,4,5]. This prompted us to conduct the current study, which has revealed that hepatectomy provided better outcomes for patients with HCC ≤ 4 cm than percutaneous ablation. This may partly be due to the fact that hepatic functional reserve was better in patients who had undergone hepatectomy. Despite this, we found that the incidence of local recurrence decreased and long-term survival increased independently after hepatectomy, indicating that

Table 4 Incidence of vascular invasion according to tumor size

| Tumor size (cm) | Vascular invasion | | P value |
|-----------------|-------------------|-----------------|---------|
| | (-) | (+) | |
| ≤ 2 | 22 | 1 ¹ | 0.033 |
| > 2 | 46 | 16 ² | |

¹Portal venous invasion was noted. ²Portal venous invasion in 10 patients, hepatic venous invasion in 3, and both portal and hepatic venous invasion in 3.

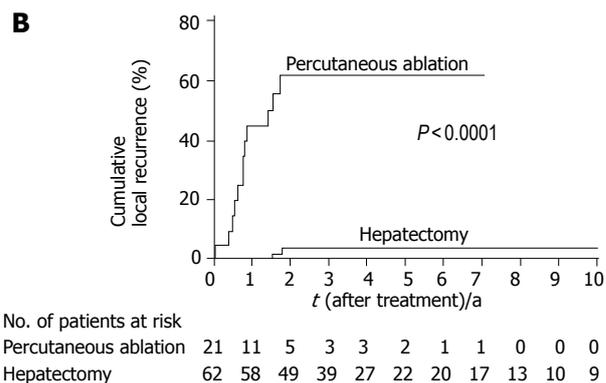
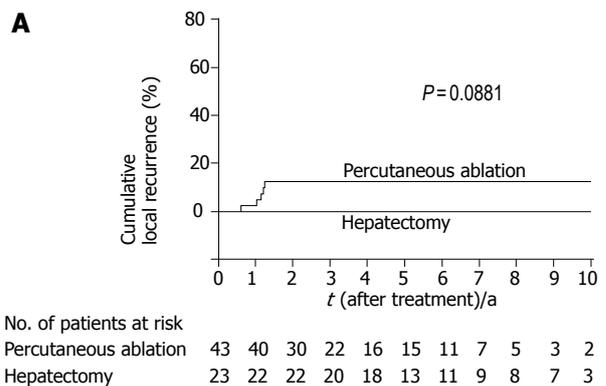


Figure 2 Kaplan-Meier estimates of local recurrence by tumor size. **A:** Among tumors ≤ 2 cm, the incidence of local recurrence reached a plateau of 12% at 15 mo after percutaneous ablation, whereas no recurrences had occurred after hepatectomy; **B:** among tumors > 2 cm, the incidence of local recurrence reached a plateau of 61% at 21 mo after percutaneous ablation, whereas it reached a plateau of 4% at 22 mo after hepatectomy.

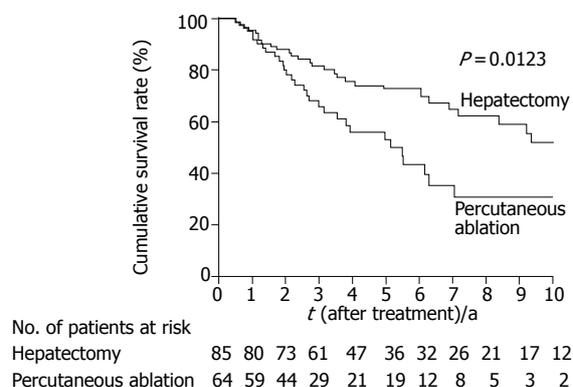


Figure 3 Kaplan-Meier estimates of survival. The median survival time was 122 mo with a 10-year survival rate of 53% in patients who had undergone hepatectomy. The median survival time was 66 mo with a 10-year survival rate of 31% in patients who had undergone percutaneous ablation.

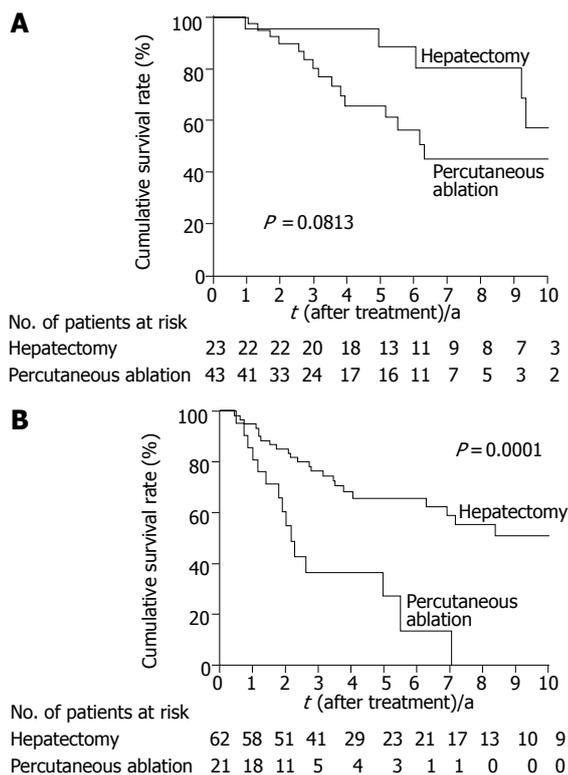


Figure 4 Kaplan-Meier estimates of survival by tumor size. **A:** Among patients with tumors ≤ 2 cm, survival was better following hepatectomy (median survival time of 122 mo; 10-year survival rate of 58%) than following percutaneous ablation (median survival time of 76 mo; 10-year survival rate of 45%); **B:** among patients with tumors > 2 cm, survival was better following hepatectomy (median survival time of 126 mo; 10-year survival rate of 51%) than following percutaneous ablation (median survival time of 26 mo; 10-year survival rate of 0%).

this is an oncologically reliable treatment modality for HCC ≤ 4 cm.

Local recurrence was found to be more frequent after percutaneous ablation than after hepatectomy. Prospective, randomized controlled trials for PEI treatment of HCC ≤ 4 cm^[23-25] demonstrated that cumulative 2-year local recurrence rates ranged from 33% to 45%. These rates are comparable with the 28% incidence of local recurrence after percutaneous ablation detected in the current study. Local recurrences after percutaneous ablation may be attributable to insufficient ablation of the primary tumor and/or the presence of portal or hepatic venous tumor thrombi in the adjacent liver^[26,27]. Hepatectomy removes a rim of non-neoplastic liver parenchyma (a resection margin) together with the primary tumor, and thus eradicates both the primary tumor and venous tumor thrombi within the resection margin. This may explain better the outcomes following hepatectomy. In the current study, the beneficial effect of hepatectomy was prominent in a subgroup of patients with tumors > 2 cm (Figures 2B and 4B), suggesting that these patients are good candidates for hepatectomy. Tumors > 2 cm had a higher incidence of vascular invasion than tumors ≤ 2 cm (Table 4). Taken together, these findings suggest that the beneficial effect of hepatectomy is due to the clearance of venous tumor thrombi in the adjacent liver in addition to the complete removal of the primary tumor.

HCC mainly spreads *via* the portal and hepatic veins.

Vascular invasion is an established adverse prognostic factor of HCC^[19-21,28-30], and the incidence of vascular invasion increases as the tumor enlarges^[30-33]. The current study confirmed this, with vascular invasion being more frequent in tumors > 2 cm. Recent authors have suggested that tumors > 2 cm are independently associated with local failure after RFA^[34,35]. Considering that vascular invasion was less frequent in tumors ≤ 2 cm in our patients, percutaneous ablation appears to be an appropriate treatment modality for HCCs ≤ 2 cm. Despite this, outcomes for patients with HCCs ≤ 2 cm were better following hepatectomy than following percutaneous ablation. Due to the small sample size of this study, however, these differences were only marginally significant. We believe that hepatectomy may also be an appropriate treatment modality for HCCs ≤ 2 cm, provided that the patient is robust and that the hepatic functional reserve of the patient is at a level permitted for the resection.

Recent evidence suggests that high pre-treatment serum alpha-fetoprotein levels are associated with both the presence of portal venous invasion and intrahepatic recurrences after the treatment in HCC^[26,36-41]. Serum alpha-fetoprotein levels were independently associated with local recurrences in the current study. The above findings suggest that high serum alpha-fetoprotein levels predict the presence of portal venous invasion, which may lead to treatment failure, in patients with HCC.

The current study has some limitations. First, it was a retrospective analysis of a small number of patients; second, the follow-up period in 64 patients was < 60 mo; and third, percutaneous ablation therapy included three different treatment modalities. However, we believe that these limitations do not significantly influence the outcome of the study, as the marked differences between each group appear to overcome these biases.

In conclusion, hepatectomy provides both better local control and better long-term survival for patients with HCC ≤ 4 cm than percutaneous ablation, probably because hepatectomy eradicates both the primary tumor and venous tumor thrombi within the hepatectomy margin. Among the patients with HCC ≤ 4 cm, those with tumors > 2 cm are good candidates for hepatectomy, provided that the level of hepatic functional reserve of the patient is suitable for resection.

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Preoperative assessment of vascular anatomy of inferior mesenteric artery by volume-rendered 3D-CT for laparoscopic lymph node dissection with left colic artery preservation in lower sigmoid and rectal cancer

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Abstract

AIM: To determine the distance between the branching point of the left colic artery (LCA) and the inferior mesenteric artery (IMA) by computed tomography (CT) scanning, for preoperative evaluation before laparoscopic colorectal operation.

METHODS: From February 2004 to May 2005, 100 patients (63 men, 37 women) underwent angiography performed with a 16-scanner multi-detector row CT unit (Toshiba, Aquilion 16). All images were analyzed on a workstation (AZE Ltd, Virtual Place Advance 300). The distance from the root of the IMA to the bifurcation of the LCA was measured by curved multi-planar reconstruction on a workstation.

RESULTS: The IMA could be visualized in all the cases, but the LCA was missing in two patients. The mean distance from the root of the IMA to the root of the LCA was 42.0 mm (range, 23.2-75.0 mm). There were no differences in gender, arterial branching types, body weight, height, and body mass index.

CONCLUSION: Volume-rendered 3D-CT is helpful to assess the vascular branching anatomy for laparoscopic surgery.

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Key words: Laparoscopy-assisted colorectal surgery; Multi-detector row CT angiography; 3D-CT; Inferior mes-

INTRODUCTION

Laparoscopy-assisted colorectal surgery is now an important surgical modality for colorectal neoplasms. For left side colon cancer, the root of the inferior mesenteric artery (IMA) is usually cut for D3 lymph node dissection. We keep the left colic artery (LCA) intact even in D3 dissection, to maintain the blood supply of the preserved proximal sigmoid colon (manuscript submitted for publication). However, it is at times difficult to locate the root of the LCA when dissecting around the IMA.

Helical computed tomography (CT) and multi-detector CT produce an image of the mesenteric vasculature with faster scanning and narrower collimation^[1] than that previously obtained by conventional angiography. Recently, three-dimensional (3D) angiography was shown to help the preoperative assessment of vascular anatomy for laparoscopic lymph node dissection. In this study, we have used preoperative 3D-CT to investigate the vascular anatomy of the IMA for laparoscopic lymph node dissection around IMA with the preservation of LCA, for rectal cancer.

MATERIALS AND METHODS

Patients

One hundred patients (63 men, 37 women) who underwent contrast-enhanced CT from February 2004 to May 2005 were included (Table 1).

Table 1 Medical conditions of patients examined in this study

| Medical conditions | n |
|-------------------------------------|-----------|
| Aortic aneurysm | 32 |
| Arteriosclerosis | 26 |
| Varicose veins | 5 |
| Takayasu's arteritis | 3 |
| Angina pectoris | 1 |
| Myocardial infarction | 1 |
| Cerebral infarction | 1 |
| Hypertension | 1 |
| Thrombosis | 4 |
| Total cardiovascular disease | 74 |
| Colon cancer | 9 |
| Hepatocellular carcinoma | 5 |
| Pancreatic cancer | 3 |
| Malignant lymphoma | 2 |
| Esophageal cancer | 1 |
| Total neoplasm | 20 |
| Myoma uteri | 3 |
| Pancreatic cyst | 2 |
| Cholecystolithiasis | 1 |
| Total others | 6 |

Table 2 Distance from IMA root to LCA root and branching types of IMA

| Sex | n | Age (range)/yr | Distance (range)/mm | 98 patients with branching type | | | |
|--------|-----|----------------|---------------------|---------------------------------|----|----|----|
| | | | | A | B | C | D |
| Male | 63 | 68.7 (30-89) | 41.8 (25.3-73.5) | 6 | 28 | 21 | 7 |
| Female | 37 | 67.5 (20-90) | 42.3 (23.2-75.0) | 4 | 17 | 6 | 9 |
| Total | 100 | 68.3 (20-90) | 42.0 (23.2-75.0) | 10 | 45 | 27 | 16 |

LCA was missing in two patients (1 male and 1 female). D: Branching type undetermined.

Table 3 Distance from IMA root to LCA root in different branching types

| Branching type | Mean distance (mm) (SD) |
|----------------|-------------------------|
| Type A | 44.1 (7.36) |
| Type B | 42.2 (10.2) |
| Type C | 38.3 (7.41) |
| All (n=82) | 41.3 (9.15) |

Multi-detector row CT angiography

Multi-detector row CT angiography was performed in all the patients with 16-detector row scanners (Toshiba, Aquilion 16). Patients received 2 mL/kg (not exceeding 150 mL in total) of nonionic contrast medium (300 g/L) injected at a rate of 3 mL/s through a cubital vein, using an 18-22-gauge catheter and a power injector. Scanning was initiated by an automatic bolus-tracking system. The circular region of interest was placed in the abdominal aorta. The threshold level was 100 Hounsfield units. The CT scan parameters were collimation 16 mm×1 mm, helical pitch 23. Images were reconstructed at 1-2 mm thickness. All images were analyzed on a workstation (AZE Ltd, Virtual Place Advance 300). The 3D-volume rendering and curved multi-planar reconstruction were done on a workstation by two radiologists. The length from the root of IMA to bifurcation of LCA was measured by curved multi-planar reconstruction.

Statistical analysis

Chi-square test was applied to analyze the differences in incidence of the types of branching of LCA, sigmoidal artery (SA), and SRA. Pearson's product moment test was used to estimate correlation between the distance from the root of IMA to LCA and body weight, height, and body mass index (BMI), respectively.

RESULTS

There was no age difference between male (mean, 68.7 years; range, 30-89 years) and female (mean 67.5 years; range, 20-90 years) patients; the mean overall age was 68.3 years (range, 20-90 years). The IMA was observed on 3D-CT in all the patients analyzed. In two patients, 3D-CT demonstrated that the LCA was missing. The mean distance from the root of IMA to the root of LCA for all cases was 42.0 (range, 23.2-75.0) mm; gender differences were not observed [41.8 (range, 25.3-73.5) mm for male

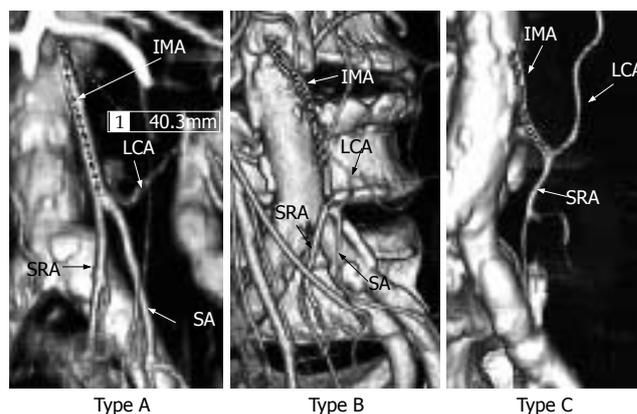


Figure 1 The branching types of IMA, LCA and SA. Type A: the three arteries branch off from the same point; Type B: SA branches off from LCA; and Type C: SA branches off from SRA.

patients vs 42.3 (range, 23.2-75.0) mm for female patients].

The branching of LCA, SA, and SRA could be determined in 82 cases (Table 2). Three types of branching were seen (Figure 1): Type A, where the three arteries branch off from the same point (10 cases: 6 males, 4 females); Type B, where SA branches off from LCA (45 cases: 28 males, 17 females); and Type C, where SA branches off from SRA (27 cases: 21 males; 6 females). There was no significant difference in the type of branching between male and female patients ($P=0.293$; Table 2). The distances from IMA root to LCA root (43.1 ± 9.2 mm, $n=82$) in Types A, B, and C were 44.1 ± 7.4 mm, 42.2 ± 10.2 mm, and 38.7 ± 7.4 mm, respectively. The distance in Type C was apparently shorter than in Type A, ($P=0.07$), and there was no significant difference between the three types (Table 3).

The distance from IMA root to LCA root was examined in relation with height and body weight measured in 76 and 77 patients, respectively. There was no correlation between this distance and height ($r=0.139$, Figure 2A), body weight ($r=0.190$, Figure 2B), or BMI ($r=0.102$, Figure 2C).

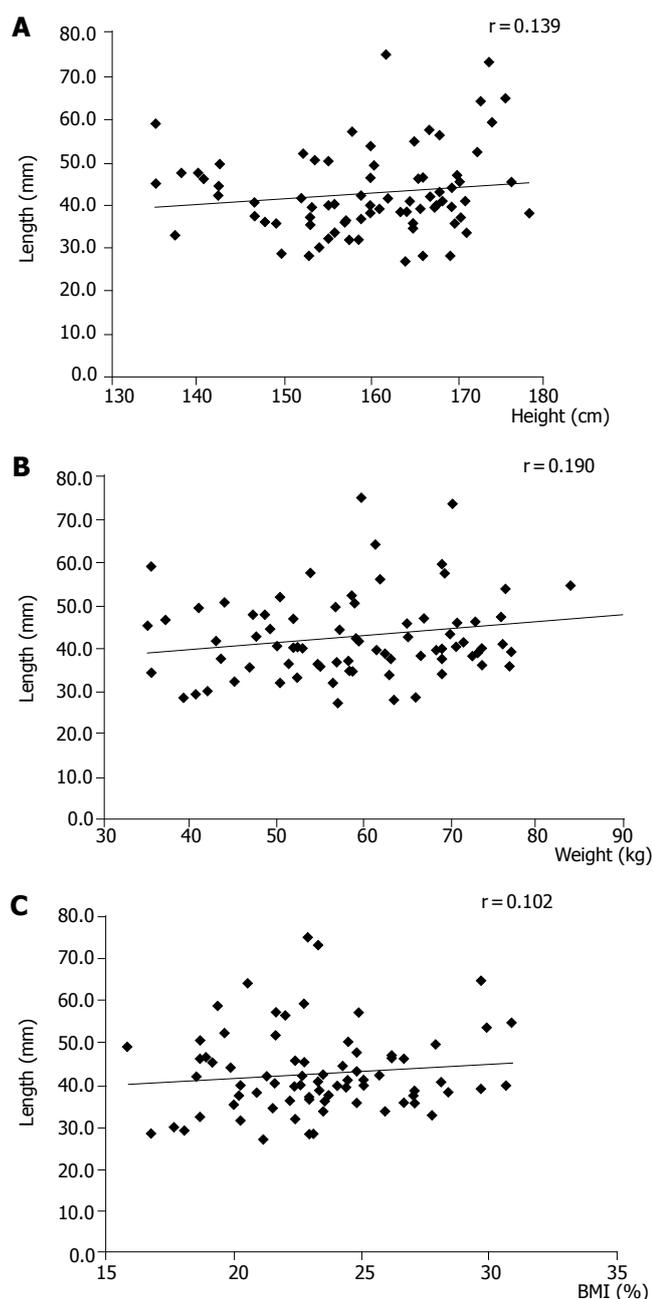


Figure 2 The distance from IMA root to LCA root does not correlate with patient's height (A), weight (B), or BMI (C).

DISCUSSION

Recent advances in multi-detector row CT combined with rapid intravenous administration of contrast material have allowed excellent opacification of the mesenteric vessels. Attempts have been made to use this modality to obtain preoperative information on mesenteric vasculatures^[2-4]. The blood supply and lymphatic system of the colon are simple compared with those of the stomach. Accordingly,

D3 lymph node dissection for sigmoid and rectal cancer is performed by cutting the root of the IMA. In contrast, for gastric cancer, the lymph nodes around the common hepatic artery, celiac axis, and splenic artery, most of which are group 2 lymph nodes, should be dissected while preserving these major vessels. We have reported a D3 lymph node dissection technique around IMA for lower sigmoid colon or rectal cancer cases, whereby the LCA is kept intact to maintain the blood supply of the preserved proximal sigmoid colon (manuscript submitted for publication). LCA is absent in 12% of individuals, in whom the colosigmoid artery performs its function^[5,6]. In some cases, the length from the root of IMA to that of LCA is fairly long and lymph node dissection around the IMA can be a stressful procedure until the LCA is located, particularly in laparoscopic surgery. To help in locating the LCA during laparoscopic surgery, we have conducted a preoperative 3D-CT investigation to examine the branching of LCA, SA, and SRA, and evaluate the distance from the root of IMA to that of LCA.

In our study, there were no gender differences in mean distance and distance ranges. There was also no correlation between body weight, height, or BMI and the distance from IMA root to LCA root. In branching Type C, the distance from IMA root to LCA root tended to be shorter, but this was not statistically significant. Thus, the distance between IMA root and LCA root is independent of the type of branching or the patient's constitution. However, volume-rendered 3D-CT helped identify vascular branching types for laparoscopic surgery and indicated that the distance of the branching point of the LCA from the IMA root may range from as short as 20 mm to sometimes longer than 70 mm.

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VIRAL HEPATITIS

High serum leptin is an independent risk factor for non-response patients with low viremia to antiviral treatment in chronic hepatitis C

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predictor of response to antiviral treatment in chronic hepatitis C with low viremia.

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Abstract

AIM: To determine whether body weight and/or serum leptin were independent predictors of response to antiviral treatment in patients with chronic hepatitis C.

METHODS: A retrospective evaluation was performed in 139 patients with chronic hepatitis C treated with interferon (IFN) from 1996 to 2000. Sustained response was defined as negative by hepatitis C virus (HCV) RNA analysis using PCR and normal transaminase at 24 wk after cessation of IFN therapy. Patients who remained positive for HCV RNA at the end of IFN treatment were defined as resistant to IFN therapy. Sex, age, body mass index (BMI) (≥ 25 vs < 25), complication of diabetes mellitus, serum leptin level (≥ 8.0 $\mu\text{g/L}$ vs < 8.0 $\mu\text{g/L}$), and the stage of liver fibrosis by needle biopsy (F1/F2 vs F3/F4) were examined.

RESULTS: Sustained response was achieved in 33 patients (23.7%), while others failed to show a response to IFN therapy. Overall, the factors associated with sustained antiviral effects were HCV-RNA load, HCV genotype, serum leptin level, and stage of liver fibrosis evaluated by univariate analysis. BMI was not associated with any therapeutic effect of IFN. Multivariate analysis indicated that HCV-RNA load was a significant risk factor, but among the patients with low viremia (HCV-RNA < 100 MU/L), leptin level was an independent risk factor for IFN resistance. Namely, a high level of serum leptin attenuated the effect of IFN on both male and female patients with low viremia.

CONCLUSION: High serum leptin level is a negative

INTRODUCTION

Hepatitis C virus (HCV) usually causes chronic infection, which can result in chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC)^[1]. Since interferon (IFN) therapy including pegylated interferon with ribavirin has been introduced, antiviral therapy is able to induce viral clearance and marked biochemical and histological improvements^[2]. The response to IFN therapy varies among different viral (viral load and particularly genotype) and host factors, which may include race, sex, age, obesity, and presence of liver fibrosis. In Japan, around 70% of patients with chronic hepatitis C are infected with the HCV genotype 1b, and around 25% have genotype 2a. Sustained virological response to IFN monotherapy is as low as 10-20% in genotype 1b infections, whereas the response is more than 60% in genotype 2 infections until the combination therapy with pegylated interferon and ribavirin were presented^[3,4]. However, it is not still possible to predict the response to IFN therapy in individual patients with each HCV genotype.

Obesity, a modifiable risk factor, may be an important cofactor in both accelerating fibrosis and increasing liver necroinflammatory activity in chronic hepatitis C^[5]. Several studies have shown that obesity may also have a deleterious effect on the treatment response to both pegylated and standard IFN monotherapy^[6,7]. Recent studies have indicated that food intake and body weight are regulated by leptin, of which synthesis is predominantly localized

in adipose tissue^[8]. The circulating leptin concentration correlates with fat mass size and distribution^[9]. In addition to regulating food intake and energy expenditure, leptin has other metabolic effects on peripheral tissue, including modulation of action of insulin and in wound healing^[10]. Activated rat hepatic stellate cells express leptin, and rat sinusoidal endothelial and Kupffer cells express the signaling-component isoform of the leptin receptor. Exposure of these cells to leptin results in an increased expression of transforming growth factor- β (TGF- β), which, in turn, stimulates fibrogenesis in hepatic stellate cells^[11]. Recent reports have indicated that inflammatory cytokines, including TNF- α or IL-1, determine both leptin expression and circulation^[12,13].

Judging from these results, besides viral factors, those related to individual patients including leptin and/or obesity might have important effects on IFN therapy. In this study, we have evaluated the risk factors that were related to resistance to IFN therapy in patients with chronic hepatitis C.

MATERIALS AND METHODS

Materials

One hundred and thirty-nine patients with chronic hepatitis C, who had received IFN monotherapy from 1996 to 2000 at Saga Medical School Hospital, were selected according to the following inclusion criteria: persistently elevated alanine aminotransferase (ALT) levels; positive for anti-HCV (third-generation enzyme immunoassay, Chiron Corp., Emeryville, CA, USA); positive for HCV RNA qualitative analysis using PCR (nested polymerase chain reaction or Amplicor, Roche Diagnostic Systems, CA, USA); liver biopsy consistent with chronic hepatitis examined within 6 mo of enrolment; and naïve to IFN therapy. Exclusion criteria for treatment were as follows: nearly decompensated cirrhosis and/or HCC; positive for hepatitis B surface antigen (radioimmunoassay, Dainabot, Tokyo, Japan); autoimmune liver disease; hemochromatosis; Wilson's disease; primary biliary cirrhosis; ingestion of more than 40 g/d of alcohol within the previous year; history of uncontrolled depression or psychosis; and patients with uncontrolled diabetes.

The data of patients' sex, age, body mass index [BMI: weight (kg)/height (m²)], HCV genotype, HCV qualitative analysis with PCR, 75 g oral glucose tolerance test (75 g OGTT), serum leptin at initiation of treatment, and ethanol consumption were examined. Serum leptin was measured by a radioimmunoassay (RIA, Linco Research Inc., St. Louis, MO, USA). Serum was collected at the initiation of treatment following an overnight fast for 8 h.

Liver histopathological examination

Liver biopsy specimens were obtained percutaneously or at peritoneoscopy using a modified Vim-Silverman needle. Liver biopsy specimens were reviewed by a single hepatologist, who was blinded to the clinical information of the subjects. For each liver biopsy specimen, hematoxylin-eosin and silver impregnation for collagen were available. Chronic hepatitis was diagnosed based

on histopathological assessment according to a scoring system that includes semi-quantitative assessment of liver disease grading and staging^[14]. Steatosis was evaluated by Adinolfi grading system in which steatosis is graded from 0 to 4 based on the percentage of hepatocytes involved as follows: 0 = none involved; 1 = up to 10%; 2 = up to 30%; 3 = up to 60%; and 4 = more than 60%^[15].

IFN therapy

In this protocol, 41 patients (29.5%) received IFN- β every day for 6 or 8 wk, and 98 patients (70.5%) received IFN- α for 24 wk (every day for 2 wk, followed by three times per week for 22 wk). A median total dose of IFN was 27 720 MU (range: 25 200-28 800 MU) in those treated with IFN- β and 62 500 MU (range: 46 800-78 000 MU) in those treated with IFN- α . Sustained response (SR) was defined as negative by HCV RNA qualitative analysis using PCR and normal ALT at 24 weeks after cessation of IFN therapy. Patients who remained positive for HCV RNA by PCR during and at the end of IFN treatment were defined as resistant to IFN therapy (non-response: NR). The study protocol was approved by the Human Ethics Review Committee of Saga Medical School Hospital and informed consent was obtained from each subject.

Evaluation of patients

Patients were divided into two groups: SR and NR. All were classified by age, sex, HCV RNA (low: <100 MU/L; and high: \geq 100 MU/L), viral serotype, alcohol intake, complication of diabetes (evaluated by 75 g OGTT), BMI (non-obese: <25; obese: \geq 25), leptin level (high: \geq 8 μ g/L; low: <8 μ g/L), and pathological findings of biopsy samples.

Statistical analysis

Comparisons between the two groups regarding these factors were performed using the χ^2 test. Parameters that had an influence on IFN-response were compared by the univariate Cox's proportional hazard model analysis. Variables that achieved statistical significance by univariate analysis were subsequently included in a multivariate proportional hazard model analysis. All analyses were carried out using SAS program (SAS Institute, Cary, NC, USA). Differences were considered significant if $P < 0.05$. Results were expressed as mean \pm SD unless otherwise stated.

RESULTS

Background characteristics of patients at the start of IFN monotherapy

Subjects included 87 men and 52 women. Mean BMI was 23.4 kg/m², ranging from 16.9 to 33.6 kg/m². Six percent of patients had an alcohol intake of more than 40 g/d. Ten subjects were complicated with diabetes mellitus. Seventy-one percent of patients were infected with HCV serotype 1, whereas 29% were infected with serotype 2. The subjects were divided into two groups using a cut-off viral load of 100 MU/L, and as a result, 50 subjects (16 women) showed a low viremia level (<100 MU/L), and 89 (36 women) a high viremia level (>100 MU/L).

Table 1 Demography of chronic hepatitis C patients and comparison between NR and SR to interferon

| | Total (n = 139) | NR (n = 106) | SR (n = 33) | P value |
|---|--------------------|-----------------|----------------|---------|
| Age (yr) | 49.9±11.1 | 70:30 | 25:6 | NS |
| Sex (male:female) | 87:54 | 62:44 | 25:8 | NS |
| HCV RNA load (<100 U/L:≥100 U/L) | 50:89 | 23:83 | 27:6 | <0.0001 |
| Serotype (1:2) | 99:40 | 81:25 | 18:15 | 0.026 |
| Alcohol intake (yes:no) | 8:131 | 6:100 | 2:31 | NS |
| Complication of diabetes mellitus (yes:no) | 10:129 | 6:100 | 4:29 | NS |
| Body mass index (<25:≥25) | 108:31 | 80:26 | 28:5 | NS |
| Leptin (<8 µg/L:≥8 µg/L) | 91:48 | 63:43 | 25:3 | 0.017 |
| Pathological findings | | | | |
| Grading (A0, A1:A2, A3) | 28:111 | 18:87 | 9:23 | NS |
| Staging (F0, F1:F2, F3) | 52:87 | 34:71 | 1:17 | 17:15 |
| Steatosis (grade 1, 2,3, 4) | 93:46 | 69:36 | 23:9 | NS |

NR: Non-responder; SR: Sustained responder.

Liver histopathological examination revealed: 28 subjects with mild activity (A₀ plus A₁) and 111 with severe activity (A₂ plus A₃); 52 with mild fibrosis (F₀ plus F₁) and 87 with advanced fibrosis (F₂ plus F₃); 93 with mild steatosis (steatosis grade 1 plus 2) and 46 with severe steatosis (grade 3 plus 4). Mean serum leptin concentrations in all the subjects was 6.8 ± 5.5 µg/L, while high serum leptin concentrations beyond 8 µg/L were detected in 48 (34.5%) patients (Table 1).

Univariate analysis between SR and NR groups

SR was achieved by 33 patients (23.7%), while the remaining 106 patients (76.3%) failed to show a response. Comparison between the two groups evaluated by χ^2 is shown in Table 1. Low viremia level was more frequent in patients with SR than in those with NR ($P < 0.0001$). In addition, HCV serotype was related to the response to IFN ($P = 0.026$). Subjects with low serum leptin levels (<8 µg/L) were significantly associated with a good IFN-response ($P = 0.017$), whereas the low viremia group with a high serum leptin level (≥8 µg/L) achieved SR only in 12.5% of the subjects.

When subjects were classified into mild fibrosis (F₀+F₁) and advanced fibrosis (F₂+F₃), subjects with mild fibrosis were more likely to have a good response to IFN ($P = 0.038$). There was no significant difference of BMI or hepatic steatosis grade or leptin/BMI ratio (data not shown) between NR and SR. These factors were evaluated by univariate analysis and four possible risk factors that influenced the effect of IFN therapy on HCV virus were selected: serotype, HCV-RNA, liver fibrosis, and serum leptin level (data not shown).

Multivariate analysis between SR and NR groups

Multivariate analysis indicated that viremia level was the most significant factor among the four selected ones (Table 2). As a next step, we evaluated the data limited to the factors in the low viremia patients ($n = 50$). In patients with low viremia, only leptin level was an independent risk factor for IFN resistance (Table 2). This result regarding leptin level was supported by analysis using another

Table 2 Multivariate analysis of parameters

| | | P | Odds ratio | RR (95%CI) |
|---------------------------------------|--------------|---------|------------|--------------|
| independently | Serotype | 0.93 | 1.05 | (0.34-3.32) |
| influenced SR (n=33) | HCV RNA | <0.0001 | 12.74 | (4.15-39.08) |
| | Fibrosis | 0.18 | 0.49 | (0.18-1.39) |
| | Leptin level | 0.14 | 2.87 | (0.18-11.63) |
| 50 subjects with low viremia level | Serotype | 0.95 | 0.25 | (0.25-3.63) |
| | Fibrosis | 0.14 | 0.37 | (0.1-1.4) |
| | Leptin level | <0.05 | 9.38 | (0.99-88.85) |

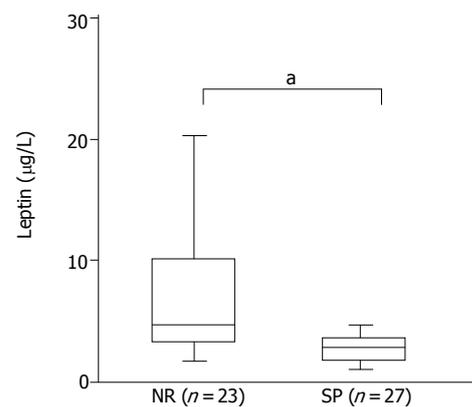


Figure 1 Effectiveness of IFN and serum leptin level in subjects with a low viremia level. NR: Non-responder group for IFN therapy; SR: Sustained responder group. ^a $P < 0.05$, NR vs SR. Mann-Whitney *U*-test.

statistical method (Figure 1), that is, evaluated by *U*-test. In patients with low viremia, leptin levels were significantly higher in the NR group compared to the SR group ($P < 0.05$).

Although histopathological findings were not independent risk factors in multivariate analysis for IFN therapy, there were significant relations between the leptin level and activity grading + steatosis evaluated by Kruskal-Wallis test (Figures 2A and 2B). Evaluations using Kruskal-Wallis did not indicate a relationship between liver fibrosis and serum leptin level (Figure 2C).

DISCUSSION

This study demonstrated the possibility that among the host factors, serum leptin level can be a significant negative predictor for IFN-response in chronic hepatitis C. We evaluated 139 naïve patients with chronic hepatitis C who had received IFN monotherapy at our hospital. In this protocol, IFN- α or IFN- β was administered because intramuscular IFN- α and once-a-day intravenous IFN- β resulted in a similar sustained HCV RNA clearance in patients with chronic hepatitis C in Japan^[16]. We showed the response rate and pretreatment predictive factors associated with IFN response in these patients. It was suggested that serum leptin level was an important factor for IFN response, although viral load was most critical for IFN therapy.

Overall, the SR rate in our study was 23.7% in patients receiving IFN monotherapy. Subjects with a low serum leptin level (<8 µg/L) were significantly associated with

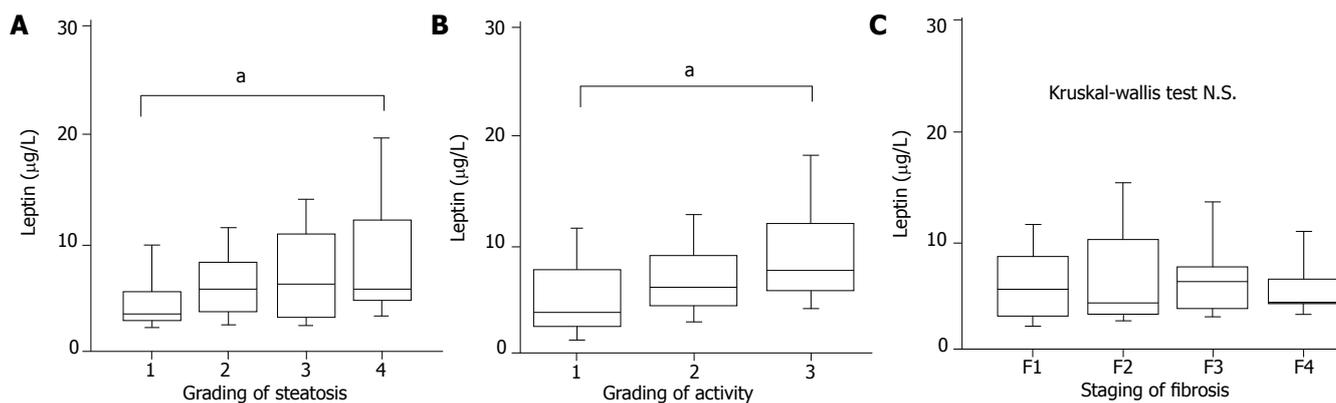


Figure 2 Histopathological parameters and serum leptin level. **A:** Hepatic steatosis and serum leptin level. Steatosis was evaluated by the Adinolfi grading system in which steatosis is graded from 0 to 4 based on the percentage of hepatocytes involved. There were significant relationships between leptin level and hepatic steatosis evaluated by Kruskal-Wallis test ($^bP < 0.01$, steatosis 1 vs 2 vs 3 vs 4); **B:** Necroinflammatory activity and serum leptin level. There were significant relationships between leptin level and activity grading evaluated by Kruskal-Wallis test ($P < 0.05$). The scoring system includes the semi-quantitative assessment of liver disease grading of histopathological inflammatory activity (1: mild, 2: moderate, 3: severe activity; $^aP < 0.05$, activity 1 vs 2 vs 3); **C:** Fibrosis and serum leptin level. F1: Fibrosis is located to the portal tract; F2: fibrosis is located septally with distinct widening of portal fields; F3: fibrosis extended portally to the portal; F4: liver cirrhosis. There was no relationship between leptin level and liver fibrotic staging evaluated by Kruskal-Wallis test. NS: not significant.

a good IFN response. Multivariate analysis of all factors could not identify serum leptin level as an independent predictive factor associated with SR, because the high viral load markedly influenced on IFN therapy as has been indicated in several previous studies^[17,18]. When evaluation was limited in the 50 patients with low viral load (< 100 MU/L), multivariate analysis revealed a significantly independent association between high serum leptin level and low IFN-response (OR = 9.38, $P < 0.05$). In this study, the relationship between BMI and serum leptin level was clearly seen, but not BMI alone, as an independent risk factor for IFN therapy. The reason for this discrepancy between leptin level and BMI was not ascertained in this study, but most Japanese obese subjects have a moderate BMI of around 25^[19].

A histopathological approach indicated that serum leptin level was significantly associated with worsening steatosis and inflammatory activity, although these two factors in liver samples were not independent risk factors for IFN therapy in this study. Piche *et al.*^[20] reported that leptin is an independent metabolic factor associated with the severity of liver fibrosis in patients with chronic hepatitis C and higher BMI. Previous study demonstrated that overweight patients with chronic hepatitis C had significantly more steatosis and increased serum insulin and leptin levels^[21]. On the other hand, Giannini *et al.*^[22] demonstrated that there was no relationship between serum leptin levels and the severity of steatosis, but their study design was different from this study. Namely, they excluded patients who had complicated diabetes mellitus, hyperlipidemia, obesity, and alcohol ingestion. The relationship between serum leptin levels and the severity of steatosis in patients with chronic hepatitis C remains controversial, because serum leptin levels can be affected by the progression of chronic hepatitis and host factors, such as visceral adiposity, insulin resistance and other adipokines. In this study, we could not demonstrate the relationship between liver fibrosis and serum leptin; the reason for this result might be explained by the fact that we enrolled naïve

patients for IFN therapy in this study.

The mechanism of leptin in the antiviral response to IFN treatment was not determined in this study, but several studies might support the possibility of a relationship between the two factors. Serum leptin concentration increased in liver steatosis and cirrhosis in addition to obesity^[23]. Another study also has shown that liver fibrosis correlates with steatosis and obesity^[24]. From these studies, it could be judged that lipid deposits within the hepatocytes might cause functional disturbances by increasing the architectural distortion of the hepatic lobule caused by fibrosis, and decrease the contact area between drugs (e.g., IFN) and hepatocytes. While the expression of leptin in adipose tissue increased in response to feeding and energy repletion, leptin had a proinflammatory and/or profibrogenic role that might regulate cytokine-influenced repair and fibrogenesis of liver^[11].

Our data suggests that low serum leptin might be a predictor for sustained viral response following a course of IFN therapy for chronic hepatitis C with low viremia. In this study, we did not assess the difference of serum leptin levels in males and females because it was difficult to define normal ranges of serum leptin level in various stages of hepatic fibrosis and also the activity of leptin in each male and female patients are unclear. However, high serum leptin level is a remarkable risk factor for antiviral therapy in chronic hepatitis C, because this study showed that serum leptin levels over 8 µg/L or less than 8 µg/L were able to be the threshold line for both males and females about the efficacy of antiviral therapy. This study does not address the issue if pretreatment intervention to decrease serum leptin level before IFN therapy would compensate for the low response rate in patients with a high serum leptin level. Prospective studies are needed to investigate the effectiveness of decreasing leptin level by interventions, such as weight reduction before IFN-therapy for enhancing antiviral responsiveness, and to investigate using combination therapy by peg-interferon and ribavirin.

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Long-term lamivudine treatment for chronic hepatitis B in Japanese patients: A project of Kyushu University Liver Disease Study

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Abstract

AIM: To determine the efficacy of long-term lamivudine treatment of a large number of Japanese patients with chronic hepatitis B.

METHODS: In this retrospective, multi-center trial, 318 Japanese patients with chronic hepatitis B received 100 mg of lamivudine daily for up to 36 (median 21) mo. Virological response was a decline to a serum HBV DNA level less than 3.7 log copies/mL. Virological

breakthrough was defined as the reappearance of a serum HBV DNA level to more than 10-fold the minimum during treatment.

RESULTS: Lamivudine produced virological response in 86.8% of the 318 patients at 6 mo, in 80.2% of 252 patients at 12 mo, in 69.2% of 133 patients at 24 mo, and in 53.6% of 28 patients at 36 mo. Forward stepwise logistic regression analysis showed an HBV DNA level less than 6.8 log copies/mL ($P < 0.0001$), HBeAg negativity ($P < 0.0001$), a platelet count of $100 \times 10^9/L$ or more ($P = 0.0162$) at baseline, and a decline of the HBV DNA level of more than 3.2 log copies/mL as compared with the baseline level at 3 mo after the start of treatment ($P = 0.0003$) to be significantly associated with virological response. Among patients with a virological response, virological breakthrough was seen in 5.3% of 19 patients who responded virologically at 1 mo, in 20.7% of 203 patients at 3 mo, in 27.5% of 51 patients at 6 mo, in 33.3% of 12 patients at 9 mo, and in 100% of 3 patients at ≥ 15 mo. A virological breakthrough was found significantly more often in patients with delayed virological response.

CONCLUSION: Lamivudine treatment could suppress serum HBV DNA in most of the tested Japanese patients. Long-term efficacy might be seen in patients without HBeAg at baseline, in the absence of cirrhosis, and in patients with a decline in HBV DNA level soon after the start of treatment.

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Key words: Hepatitis B virus; Lamivudine; HBeAg; Cirrhosis

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INTRODUCTION

Chronic hepatitis B virus (HBV) infection affects 400 million people worldwide, three-quarters of whom reside in Asia^[1]. The morbidity and mortality of chronic HBV infection are a major public health concern. Vertical transmission of HBV is the main cause of chronic HBV infection in the endemic areas of Asia. In Japan which is endemic for HBV infection, transmission can be easily prevented by vaccination of at risk-infants. The prevalence of hepatitis B surface antigen (HBsAg) carriage in the general population was reported to be less than 2%^[2]. However, in areas of high HBV endemicity, persons with HBV-related cirrhosis have an approximately threefold higher risk of hepatocellular carcinoma (HCC) than those with chronic hepatitis but without cirrhosis and a 16-fold higher risk of HCC than the carriers in whom the virus is inactive^[3]. Interferon-alpha (IFN- α) has been approved for the treatment of chronic hepatitis B^[4], but it is poorly tolerated and effective in only 20%-30% of patients. Since the late 1990s, several studies have demonstrated the effectiveness of the initially available antiviral medicine, lamivudine, for patients with chronic hepatitis B: HBV DNA suppression, normalization of alanine aminotransferase (ALT), loss of hepatitis B e antigen (HBeAg). Moreover, studies have demonstrated the improvement of hepatic histology by the administration of lamivudine compared to placebo^[5-8].

Lamivudine, an oral cytosine nucleoside analog, is the (-) - beta-enantiomer of 2',3'-dideoxy-3'-thiacytidine. HBV replicates through a pregenomic RNA intermediate. Lamivudine interferes with HBV reverse transcriptase (DNA polymerase) activity and causes chain termination of nascent viral DNA, leading to the inhibition of HBV replication^[9]. Long-term treatment with lamivudine is not an option because it leads to drug resistance in most cases^[10,11]. Lamivudine treatment, especially for chronic HBV-infected patients with cirrhosis, may also act as a bridge to more definitive treatments, such as liver transplantation. However, in several countries, including Japan, liver transplantation is not easily available because of insufficiency of donors, and even in other countries, many patients have to wait for long periods for transplantation. Although several non-Asian studies, from North America and Europe, have shown the efficacy of long-term use of lamivudine^[12,13], few studies have assessed the efficacy of long-term lamivudine treatment of a large number of Japanese patients with chronic hepatitis B.

To acquire more data on these issues, 37 Japanese liver units involved in the management of HBV-related chronic liver diseases in Kyushu, Japan cooperated in this study. The objective of the present study was to analyze the results of long-term lamivudine administration for the suppression of HBV replication and the clinical outcomes of a large number of Japanese patients with chronic hepatitis B.

MATERIALS AND METHODS

Patients

This retrospective analysis encompassed 318 Japanese

chronic hepatitis B patients (231 males and 87 females, mean age 47.8 years) on lamivudine monotherapy for up to 36 (median 21, range 9-36) mo. Clinical features from 403 HBsAg-positive patients with chronic liver diseases, who started lamivudine treatment between December 2000 and March 2004 in 37 Japanese liver units in Kyushu, were recorded in a centralized database. All patients were determined to be serum HBV DNA-positive via polymerase chain reaction (PCR) assay prior to treatment. The diagnosis of chronic hepatitis and cirrhosis was based on a liver biopsy in most patients, if unavailable, on clinical laboratory, and ultrasound data. Eighty five patients were excluded from the present analysis because of one or more of the following reasons: age below 18 years; positive for antibody to hepatitis C virus or human immunodeficiency virus type 1; diagnosis of HCC within 3 mo after enrolment; time of lamivudine treatment within 9 mo; or treatment with anticancer drugs or corticosteroid drugs for other malignancies, such as leukemia, lymphoma or autoimmune diseases. Because this was a retrospective analysis of treated patients, there were no predefined criteria for treatment withdrawal or combination treatments. Criteria for withdrawal and combination treatments after the start of the treatment were dependent upon the strategy used by the physician at each center. In the present study, follow-up was stopped for patients who discontinued lamivudine treatment or started receiving a combination treatment with IFN and lamivudine, or with adefovir dipivoxil and lamivudine.

Therapeutic protocol

The patients received lamivudine (Zeffix[®], Glaxo Smith Kline, UK) orally in a single daily dose of 100 mg. Data concerning age, sex, history of prior IFN treatment, Child-Turcotte-Pugh (CTP) score, series of serum laboratory testing of ALT, total bilirubin, albumin, HBeAg, and HBV DNA level were collected. Also, we analyzed virological (time of virological response and virological breakthrough) and biological events (time of ALT normalization, ALT breakthrough, and hepatitis flare) during the observation period. The clinical events recorded were hepatic decompensation (ascites, portal hypertensive bleeding, and hepatic encephalopathy) and liver-related death during the study period.

Biochemical and virological measurement

Quantification of serum HBV DNA was performed at each center using one of the following commercial assays according to local availability: quantitative PCR assay (Amplicor HBV Monitor, Roche Diagnostics, Mannheim, Germany), over a detection range from 2.6 (corresponding to 400 copies/mL) to 7.5 log copies/mL; or transcription-mediated amplification and hybridization protect assay (TMA-HPA, Chugai Diagnostics, Tokyo, Japan), over a detection range from 3.7 log genome equivalents (LGE)/mL (corresponding to 5000 copies/mL) to 8.7 LGE/mL. A decline of the serum HBV DNA level to less than 3.7 log copies/mL during treatment was considered as a virological response. Virological breakthrough was defined as the reappearance of a serum HBV DNA level to more

than 10-fold the minimum during treatment. We analyzed whether or not an early decline of the HBV DNA level at 3 and 6 mo after the start of the treatment was related to virological response and breakthrough.

The serum ALT, bilirubin, and albumin levels were serially determined using the standard method every month before treatment and during the treatment. The upper normal limits for the ALT level were slightly different in each facility, ranging between 30 and 40 IU/mL. Normalization with an ALT level 667 or below during the treatment was considered as a biological response. A deterioration of ALT to an abnormal level after normalization during the treatment was considered as an ALT breakthrough. A deterioration of the ALT level more than 10 times the upper limit of normal (ULN) was considered as a hepatitis flare.

Statistical analysis

Categorical variables were analyzed using χ^2 test or Fisher's exact test. The Mann-Whitney *U*-test was also used to compare responders and non-responders with regard to various characteristics, when appropriate. The Cochran-Armitage's trend test was used to determine the relationship between the increases or decreases in the virological breakthrough rates of patients with virological response. Independent factors associated with responders were studied using forward stepwise logistic regression analysis of the following variables: age at the start of treatment, sex, history of prior IFN treatment, histological staging and grading, pretreatment laboratory data, serum pretreatment HBV DNA level, and the median declines of HBV DNA level at 3 and 6 mo after the start of the treatment. Forward stepwise logistic regression analysis was performed using a commercially available software package (BMDP Statistical Software Inc., Los Angeles, CA, USA) for the IBM 3090 system computer. The BMDP program LR was used to evaluate the relationship between the clinical features and SVR. Using this method, the most significant associated variable was entered into the model. After adjusting for that variable, the next most significant variable was added to the model. Two-tailed *P* values less than 0.05 were considered statistically significant.

RESULTS

Baseline assessment

The mean age and percentage over 35 years were significantly higher in patients with cirrhosis than in those without cirrhosis, while the mean ALT level, albumin, and platelet counts were significantly higher in patients without cirrhosis than in those with cirrhosis. No significant differences in sex distribution, total bilirubin, positivity of HBeAg, or HBV DNA level were observed between these groups (Table 1). This study consisted of 173 HBeAg-positive and 145 HBeAg-negative patients with a mean pretreatment HBV DNA level of 6.8 ± 1.2 (median 7.0) log copies/mL. Concerning the relationship between HBeAg and HBV DNA level, the mean HBV DNA level was significantly higher in HBeAg-positive patients with (7.3 ± 1.1 log copies/mL) and without cirrhosis (7.2 ± 1.0 log copies/mL) as compared with HBeAg-negative

Table 1 Baseline characteristics of 318 patients with chronic HBV infection treated with lamivudine (mean \pm SD)

| Characteristics | Cirrhosis | | <i>P</i> |
|---|----------------------|-----------------------|----------|
| | No <i>n</i> = 216 | Yes <i>n</i> = 102 | |
| Number of men (%) | 154 (71.3) | 77 (75.5) | 0.5168 |
| Age (yr) | 45.0 \pm 11.1 | 53.7 \pm 9.7 | <0.0001 |
| Number with 35 yr old and over (%) | 173 (80.1) | 98 (96.1) | 0.0003 |
| ALT (IU/L) | 320.9 \pm 503.3 | 101.5 \pm 95.4 | <0.0001 |
| Total bilirubin (mg/dL) | 1.2 \pm 1.3 | 1.4 \pm 1.3 | 0.1880 |
| Albumin (g/dL) | 4.0 \pm 0.4 | 3.5 \pm 0.6 | <0.0001 |
| Platelet count (mean \pm SD) ($\times 10^4$ / μ L) | 16.2 \pm 5.3 | 9.8 \pm 4.4 | <0.0001 |
| Number of HBeAg positivity (%) | 119 (55.1) | 54 (52.9) | 0.8112 |
| HBV-DNA ¹ | 6.8 \pm 1.3 | 6.6 \pm 1.2 | 0.1344 |
| Lamivudine treatment (mo) | 20.2 \pm 8.9 | 21.8 \pm 9.7 | 0.1429 |

ALT, alanine aminotransferase; HBeAg, hepatitis B e antigen; SD, standard deviation ¹Logarithmic transformed copies/mL.

patients with (6.3 ± 1.2 log copies/mL) and without cirrhosis (6.2 ± 1.1 log copies/mL) (both $P < 0.0001$). No significant difference in HBV DNA level, even classified by HBeAg status, was observed between patients with and without cirrhosis.

Virological and biological efficacy during lamivudine treatment period

In analyses of an early decline of HBV DNA level by lamivudine, the mean declines of all the studied patients were 3.2 ± 1.2 (median 3.2) log copies/mL at 3 mo and 3.6 ± 1.1 (median 3.8) log copies/mL at 6 mo after the start of treatment. During the treatment period of up to 36 (median 21) mo, a virological response was found in 90.6% (288/318) patients and ALT normalization was found in 86.2% (274/318) patients. Of the 288 with virological response, 255 (88.5%) had ALT normalization. Of the remaining 30 without virological response, 19 (63.3%), who had achieved virological suppression with a low HBV DNA level of 3.7-4.0 log copies/mL by lamivudine, had ALT normalization, but 11 (36.7%) had no ALT normalization and an HBV DNA level of more than 4.0 log copies/mL.

The mean pretreatment HBV DNA level was significantly lower in patients with virological response (6.6 ± 1.2 log copies/mL) than those without virological response (7.7 ± 0.7 log copies/mL) ($P < 0.0001$). The frequency of pretreatment HBeAg positivity was significantly lower in patients with virological response (51.0%, 147/288) than those without virological response (86.7%, 26/30) ($P = 0.0004$). No significant differences in sex distribution, age, ALT level, platelet count, presence of cirrhosis, or CTP score were found between the patients with and without virological response (Table 2).

Lamivudine suppressed serum HBV DNA to less than 3.7 log copies/mL in 69.2% patients at 3 mo, in 86.8% patients at 6 mo, in 80.2% patients at 12 mo, in 69.2% patients at 24 mo, and in 53.6% patients at 36 mo. The efficacy rate of virological response decreased with the length of the treatment period of patients who received lamivudine for over 6 mo (Figure 1).

Of the 288 patients with virological response, 224

Table 2 Virological response of 318 patients with chronic HBV infection treated with lamivudine (mean±SD)

| Characteristics | Virological response | | P |
|---------------------------------------|----------------------|---------------|---------|
| | No n = 288 | Yes n = 30 | |
| Number of men (%) | 207 (71.9) | 24 (80.0) | 0.4624 |
| Age (yr) | 47.9±11.4 | 46.9 (22-73) | 0.6453 |
| Number of cirrhosis (%) | 90 (31.2) | 12 (40.0) | 0.4403 |
| Baseline laboratory data | | | |
| Total bilirubin (mg/dL) | 1.3±1.5 | 1.1±0.6 | 0.6895 |
| Albumin (g/dL) | 3.9±0.6 | 3.8±0.6 | 0.2381 |
| Platelet count (×10 ⁴ /μL) | 14.3±5.9 | 13.2±5.8 | 0.2624 |
| Number of HBeAg positivity (%) | 147 (51.0) | 26 (86.7) | 0.0004 |
| HBV-DNA ¹ | 6.6±1.2 | 7.7±0.7 | <0.0001 |

ALT, alanine aminotransferase; HBeAg, hepatitis B e antigen; SD, standard deviation ¹Logarithmic transformed copies/mL

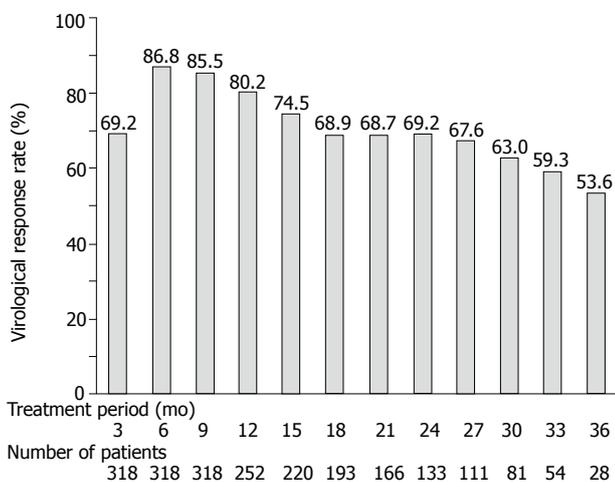


Figure 1 Treatment period and virological response rates to lamivudine treatment of Japanese patients with chronic hepatitis B virus infection.

(77.8%) had a sustained virological response, and 64 (22.2%) had a virological breakthrough. The median follow-up time was significantly shorter for patients with sustained virological response (18 mo) than those with virological breakthrough (27 mo). The frequencies of pretreatment HBeAg positivity [65.6% (42/64) *vs* 46.8% (105/224); $P=0.0123$] and cirrhosis [43.8% (28/64) *vs* 27.7% (62/224); $P=0.0218$] were significantly higher for patients with virological breakthrough than those without a breakthrough (Figure 2). No significant differences in sex distribution, age, or pretreatment HBV DNA level were observed between these groups.

HBeAg status during lamivudine treatment

Of the 318 patients, 173 (54.4%) were detected to have HBeAg in their sera at baseline. Of the 173 HBeAg-positive patients, 82 (47.4%) had clearance of HBeAg and 91 (52.6%) continued to have HBeAg during treatment. Lamivudine led to HBeAg clearance by 6.9% of the patients at 1 mo, by 24.9% of the patients at 3 mo, by 32.9% at 6 mo, by 35.8% at 9 mo, by 37.6% at 12 mo, by 45.1% at 18 mo, and by 47.4% at 36 mo, suggesting

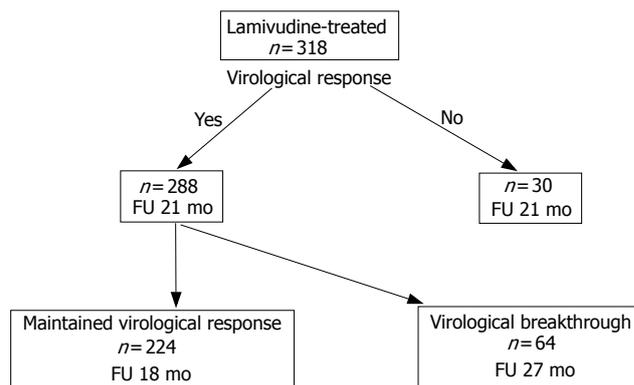


Figure 2 Virological events in all patients during lamivudine treatment period. FU: Follow-up period.

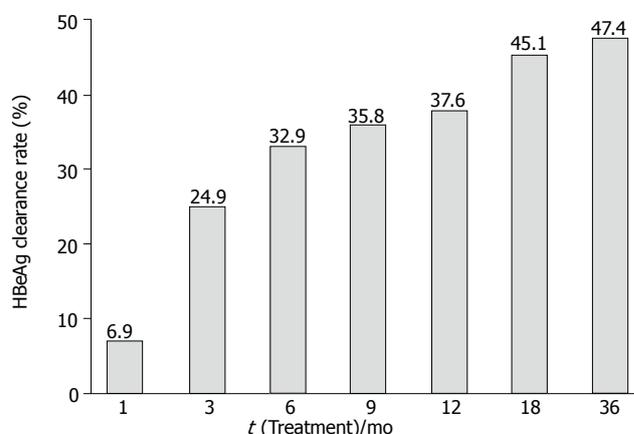


Figure 3 Relationship between treatment period and HBeAg clearance rate during lamivudine treatment of Japanese patients with chronic HBV infection.

that HBeAg clearance rates increased with the duration of lamivudine treatment (Figure 3). HBeAg clearance always occurred after virological response in all the 82 who cleared HBeAg. No significant differences in sex distribution, age, ALT level, platelet count, presence of cirrhosis, or CTP score were found between the patients with and without HBeAg clearance. Of the 145 patients with HBeAg negative at baseline, no patient reversed to HBeAg positive. We observed that the patients who cleared HBeAg (79/82, 96.3%) and the patients with HBeAg negative at baseline (141/145, 97.2%) had a significantly higher virological response rate than those without HBeAg clearance (68/91, 74.8%, $P=0.0002$, $P<0.0001$, respectively).

ALT breakthrough and hepatitis flare during lamivudine treatment

Of the 274 patients with ALT normalization by lamivudine, 231 (84.3%) had sustained ALT normalization, and 43 (15.7%) had an ALT breakthrough. Of the 43 patients with an ALT breakthrough, 4 (9.3%) had a hepatitis flare: 4 males, 3 with cirrhosis and 1 without cirrhosis, and 4 with HBeAg. However, no patient with hepatic decompensation, who had marked hyperbilirubinemia, or had a liver-related death, was observed in this study. The

Table 3 Forward stepwise logistic regression analysis for all independent factors contributing to virological response

| Factors | Odds ratio | 95% CI | P |
|---|------------|-------------|----------|
| (At baseline) | | | |
| HBV DNA less than 6.8 ¹ | 434.7 | 104.1-2000 | < 0.0001 |
| HBeAg negativity | 7.142 | 2.136-238.0 | <0.0001 |
| Platelet count more than 100x10 ⁹ /L | 4.625 | 1.242-17.22 | 0.0224 |
| (During treatment) | | | |
| Decline of HBV-DNA more than 3.2 ¹ within 3 mo of the start of treatment | 51.13 | 11.21-233.0 | <0.0001 |

HBV, hepatitis B virus; HBeAg, hepatitis B e antigen; CI, confidence interval of odds ratios ¹Logarithmic transformed copies/mL.

Table 4 Forward stepwise logistic regression analysis for all independent factors contributing to virological breakthrough

| Factors | Odds ratio | 95% CI | P |
|---|------------|-------------|--------|
| (At baseline) | | | |
| Cirrhosis | 3.527 | 1.687-7.371 | 0.0008 |
| HBeAg positivity | 2.512 | 1.265-4.989 | 0.0085 |
| Platelet counts less than 100x10 ⁹ /L | 2.386 | 1.003-5.676 | 0.0491 |
| (During treatment) | | | |
| Decline of HBV-DNA less than 3.9 ¹ within 6 mo of the start of treatment | 2.358 | 1.246-4.464 | 0.0084 |

HBV, hepatitis B virus; HBeAg, hepatitis B e antigen; CI, confidence interval of odds ratios ¹Logarithmic transformed copies/mL.

time of ALT changes always depended on the time of virological change: ALT deterioration after normalization followed an increase in the HBV DNA level in all cases. The frequency of HBeAg positivity at baseline was significantly higher in patients with a breakthrough than those without a breakthrough [72.1% (37/43) *vs* 51.5% (119/231); *P*<0.0001]. No significant differences in sex distribution, age, pretreatment HBV DNA level, presence of cirrhosis or CTP score were observed between these groups.

Relationship between early virological response and virological breakthrough

Among the 288 with virological response, virological breakthrough was seen in 1 (5.3%) of 19 who had virological response at one month, in 42 (20.7%) of 203 at 3 mo, in 14 (27.5%) of 51 at 6 mo, in 4 (33.3%) of 12 at 9 mo, and in 3 (100%) of 3 at ≥ 15 mo. Cochran-Armitage's trend test revealed that virological breakthrough was significantly more prevalent in patients with delayed virological response (*P*<0.0001) (Figure 4).

Factors contributing to virological response and breakthrough

At baseline, an HBV DNA level less than 6.8 log copies/mL (*P*<0.0001), HBeAg negativity (*P*<0.0001), and platelets count of 100x10⁹/L or more (*P*=0.0224) were significantly associated with virological response in the 318 studied patients (Table 3). Of the treatment factors, an early decline of 3.2 or more log copies/mL of HBV DNA at 3 mo after the start of the treatment was significantly associated with the response (*P*<0.0001).

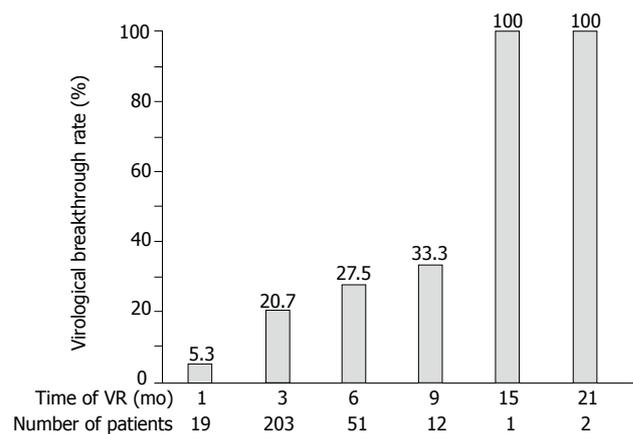


Figure 4 Relationship between time of virological response and virological breakthrough rate during lamivudine treatment of Japanese patients with chronic HBV infection. VR: Virological response.

At baseline, cirrhosis (*P*=0.008), HBeAg positivity (*P*=0.0085), and platelets count less than 100x10⁹/L (*P*=0.0491) were significantly associated with a virological breakthrough in the 288 patients with virological response (Table 4). Of the treatment factors, an early decline of 3.8 or less log copies/mL of HBV DNA at 6 mo after the start of the treatment was significantly associated with the breakthrough (*P*=0.0084).

DISCUSSION

To our best knowledge, no such large-scale studies as this of lamivudine have been carried out for Japanese chronic hepatitis B patients. In this retrospective study, good virological and biological efficacy for up to 36 mo of lamivudine treatment was seen in Japanese patients with chronic hepatitis B, with no relation to sex, age, or ALT level at baseline. The effect was sustained for the patients with HBeAg-negative before treatment, absence of cirrhosis, and with an early decline of the HBV DNA level after the start of the treatment. During the treatment, very few patients with a hepatitis flare were seen and none with hepatic decompensation, marked hyperbilirubinemia, or liver-related death were seen in this study. The aims of treatment for chronic hepatitis B are to achieve sustained suppression of HBV replication and remission of inflammation in the liver. The antiviral responses for chronic hepatitis B are categorized as biochemical (ALT normalization), virological (decrease of HBV DNA to less than 5 log copies/mL and loss of HBeAg), and histological, and as on-therapy or sustained off-therapy^[14]. Treatment for chronic hepatitis B patients seems to be necessary when the HBV DNA level exceeds 5 log copies/mL, independent of ALT activity^[11]. Lamivudine well inhibited HBV DNA replication in Japanese chronic hepatitis B patients.

HBeAg clearance usually predicts long-lasting suppression of HBV, reduced infectivity and an improved clinical prognosis^[15]. In this study, 47.4% of patients with HBeAg at baseline had HBeAg eliminated from their sera. Follow-up reports of the multicenter Asian study for Chinese patients showed that HBeAg clearance rates increased with the duration of lamivudine treatment, from 17% to 22% at

12 mo, 27 to 29% at 24 mo, and 33 to 40% at 36 mo^[12,16,17]. The results of our study were consistent with those of these non-Japanese patient, although the HBeAg clearance rates within 24 mo were relatively high in our study. Lamivudine was effective in terms of HBeAg clearance in Japanese chronic hepatitis B patients. Patients successfully treated for chronic hepatitis B are less likely to develop cirrhosis, liver failure, and HCC in comparison with those who do not respond to treatment^[18]. A randomized controlled trial of lamivudine for chronic hepatitis B patients demonstrated that HCC incidence was reduced by lamivudine antiviral therapy, showing an incidence of 3.9% in lamivudine-treated patients and 7.4% in a placebo control group, with a hazard ratio of 0.49 (95%CI = 0.25-0.99)^[19]. For chronic hepatitis B patients, antiviral therapy with lamivudine that results in sustained suppression of HBV DNA replication and hepatic necroinflammation may reduce the incidence of HCC.

It has been reported that resistance to lamivudine often develops after 6 mo of treatment^[10]. The present study was limited in its value because we detected viruses resistant to lamivudine. However, in our study, the emergence of resistant viruses could be defined by the virological breakthrough (reappearance of serum HBV DNA levels more than 10-fold increase from the minimum). A serious drawback of long-term lamivudine treatment is the development of resistant HBV mutants, i.e., the mutations in a tyrosine-methionine-aspartate-aspartate (YMDD) motif of the HBV polymerase gene, associated with increase in serum HBV DNA and the ALT level^[19]. The present study showed that the HBV DNA suppression rates by lamivudine decreased with the duration of treatment, but that a relapse of biochemical response, ALT breakthrough was found only in 15.7% of patients during these treatment periods. Lamivudine treatment withdrawal can cause HBV DNA to revert to pretreatment levels, with the relapse of clinical hepatitis^[20]. With the excellent safety and tolerability of lamivudine, continuous therapy is suggested as beneficial^[4]. After the start of phylogenetic analyses, based on inter group divergence of 8% or more over the complete HBV nucleotide sequence, seven different genotypes, arbitrarily designed A-G, have been recognized^[21,22]. Several reports have shown geographical distribution of the genotypes, with genotypes A and D predominant in Western Europe, B and C in South Asia and the Far East, and F in South America^[21-26]. Due to the geographical distribution pattern, HBV genotypes B and C are commonly observed in Japan^[24-27]. Moreover, Japan is apparently at a geographical boundary for genotypes B and C, forming a south to north gradient in which genotype C is more frequent in the south of Kyushu, and genotype B is more frequent in the north of Tohoku. Interestingly, however, genotype B is more frequent in Okinawa, the southern-most area of Japan^[27]. Our previous epidemiological study of the Japanese HBV genotype distribution showed that 95% of the patients studied had genotype C^[24]. Genotype C has been reported to cause more severe liver damage and to have lower rates of HBeAg clearance, which usually indicates cessation of HBV replication and represents a later stage of chronic HBV infection, than genotype B in Japanese patients^[24-26]. Accordingly, our results were equivalent in

the response to lamivudine to Japanese HBV genotype C patients, although we did not determine the genotyping of our patients.

Another noteworthy finding of our study was that predictive marker of the efficacy to lamivudine and its durability were HBeAg negativity and a low HBV DNA level at baseline. HBV DNA reappears in serum after cessation of lamivudine treatment because HBV replication within the HBV-infected hepatocytes originates primarily from the covalently closed circular DNA (cccDNA) of HBV in the liver. Lamivudine appears to have no effect on the level of cccDNA^[28]. Liver injury seems to be particularly severe and rapidly progressive in HBeAg-negative patients, but clinically significant HBV replication persists in them^[24]. Most HBeAg-negative chronic hepatitis B patients who are HBV DNA-positive harbor HBV variants with mutations in the precore or core promoter region, which can suppress synthesis of HBeAg^[11,26]. The clearance of HBeAg is perhaps a reflection of a loss of the cccDNA pool of HBV in the liver^[29]. The great concern of clinicians is that HBeAg negativity and a low HBV DNA level at baseline are significant predictive markers for lamivudine treatment in Japanese patients.

A previous report on Japanese patients showed that the emergence rate of lamivudine-resistant viruses in patients with cirrhosis was higher than those without cirrhosis^[28], suggesting that a virological breakthrough appears more frequently in patients with cirrhosis than those without cirrhosis. The present study showed that lamivudine treatment was not so effective or durable in patients with cirrhosis and low platelet counts. Clinicians should always do close monitoring or use other antiviral drugs because hepatitis flare was occasionally severe, especially in patients with cirrhosis. The present study also showed that an early virological response to lamivudine was predictive of both efficacy and durability, but a lack of an early virological response was found to predict a virological breakthrough. A high HBV DNA level reflects a greater pool of virus and a higher rate of virus replication, thereby increasing the likelihood that drug-resistant mutations will be selected. Such an early decrease of viral load after the start of lamivudine might be associated with the lack of viral resistance.

In conclusion, the present study suggests a long-term lamivudine treatment to be safe and to result in the reduction of serum HBV DNA in most Japanese patients with chronic hepatitis B. The efficacy is sustained in patients with HBeAg-negative at baseline, absence of cirrhosis, and a reduction of the HBV DNA level soon after the start of the treatment.

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BASIC RESEARCH

Attenuation of gastric mucosal inflammation induced by aspirin through activation of A_{2A} adenosine receptor in rats

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Abstract

AIM: To determine whether a specific adenosine A_{2A} receptor agonist (ATL-146e) can ameliorate aspirin-induced gastric mucosal lesions in rats, and reduce neutrophil accumulation and production of pro-inflammatory cytokines.

METHODS: Gastric lesions were produced by oral gavage of aspirin (200 mg/kg) and HCl (0.15 mol/L, 8.0 mL/kg). 4-{3-[6-Amino-9-(5-ethylcarbamoyl-3,4-dihydroxy-tetrahydro-furan-2-yl)-9H-purin-2-yl]-prop-2-ynyl}-cyclohexanecarboxylic acid methyl ester (ATL-146e, 2.5-5 µg/kg, IP) was injected 30 min before the administration of aspirin. Tissue myeloperoxidase (MPO) concentration in gastric mucosa was measured as an index of neutrophil infiltration. Gastric mucosal concentrations of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) were determined by ELISA. Also, we examined the effect of ATL-146e on tissue prostaglandin E₂ (PGE₂) production and gastric secretion.

RESULTS: Intragastric administration of aspirin induced multiple hemorrhagic erosions in rat gastric mucosa. The total length of gastric erosions (ulcer index) in control rats was 29.8±7.75 mm and was reduced to 3.8±1.42 mm after pretreatment with 5.0 g/kg ATL-146e ($P < 0.01$). The gastric contents of MPO and pro-inflammatory cytokines were all increased after the administration of aspirin and reduced to nearly normal levels by ATL-146e. Gastric mucosal PGE₂ concentration was not affected by intraperitoneal injection of ATL-146e.

CONCLUSION: The specific adenosine A_{2A} receptor agonist, ATL-146e, has potent anti-ulcer effects presumably mediated by its anti-inflammatory properties.

INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin are widely used as anti-inflammatory, analgesic agents^[1,2]. However, gastrointestinal injury is a serious adverse effect of NSAIDs, and effective strategies to protect the gastrointestinal mucosa are required. Many previous studies have investigated the mechanisms for the development of NSAIDs-induced gastric mucosal lesions^[3-5]. NSAIDs may cause gastric lesions by inhibiting cyclooxygenase (COX) and reducing prostaglandin (PG) production, but the exact pathogenic mechanism remains to be elucidated^[6]. Several investigators have reported that intraperitoneal injection of anti-neutrophil serum or immunoneutralization of adhesion molecules on neutrophils and endothelial cells significantly attenuates gastric mucosal injury induced by NSAIDs^[7,8]. Therefore, activation and infiltration of neutrophils into the stomach appear to contribute to the gastric mucosal lesions induced by NSAIDs.

Adenosine is a primordial signaling molecule that elicits numerous physiological responses in all mammalian tissues. The receptor-mediated effects of adenosine are mediated by four G protein-coupled receptors (A₁, A_{2A}, A_{2B}, and A₃)^[9,10] which are variably expressed on immune cells depending on cell type and species. A_{2A} receptors are found on bone marrow derived cells including neutrophils, monocytes, macrophages, lymphocytes, platelets, and mast cells^[11]. Activation of A_{2A} receptors on immune cells produces a series of responses that in general can be categorized as anti-inflammatory effects^[12]. It has been reported that the activation of A_{2A} receptors attenuates ischemia/reperfusion injury in the heart, lung, liver, and

kidney by reducing neutrophil accumulation, superoxide generation, inhibition of endothelial adherence, and expression of the adhesion molecules^[13-16]. Furthermore, activation of A_{2A} receptors on human monocytes and mouse macrophages inhibits secretion of the pro-inflammatory cytokines, IL-12 and TNF- α ^[17,18]. We have reported that ATL-146e reduces gastric mucosal lesions induced by water-immersion stress^[19]. On the basis of this evidence, we hypothesized that the activation of adenosine A_{2A} receptors would reduce gastric mucosal injury induced by aspirin. In this study, we used ATL-146e, a new compound that has been shown to be more potent and selective than the older A_{2A} agonist, CGS21680. In binding assays, ATL-146e was found to bind with more than 100 times higher affinity to recombinant human A_{2A} receptors (K_i = 0.2 nmol/L) than to the other three adenosine receptor subtypes, A₁, A₃ and A_{2B}^[20]. We have reported that a single intraperitoneal injection of ATL-146e into the rats could inhibit TNF- α and IL-1 β production, neutrophil accumulation in gastric injury induced by aspirin without affecting mucosal prostaglandin E₂ (PGE₂) concentration.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 250-300 g were fed on a standard laboratory diet and water *ad libitum*, and kept in cages in a temperature and humidity controlled room with a 12-h dark-light cycle before and during the experiment. Prior to the administration of aspirin, animals were deprived of food for 24 h but had free access to water. This experimental protocol was approved by the Akita University Animal Care Committee.

Chemicals

4-{3-[6-Amino-9-(5-ethylcarbamoyl)-3,4-dihydroxy-tetrahydro-furan-2-yl]-9H-purin-2-yl]-prop-2-ynyl}-cyclohexanecarboxylic acid methyl ester (ATL-146e) with its purity >99% was provided as a gift from Jayson Rieger, PhD Adenosine Therapeutics, Charlottesville, VA, USA^[21]. ATL-146e was dissolved in a small volume of dimethylsulfoxide and then diluted >100-fold with physiological saline just before the injection.

Effect of ATL-146e on aspirin-induced gastric mucosal injury

Aspirin-induced gastric injury was produced by intragastric administration of aspirin (200 mg/kg) and HCl (0.15 mol/L, 8 mL/kg). ATL-146e (2.5 or 5 μ g/kg, *n* = 5 each group) or vehicle was injected intraperitoneally 30 min prior to the administration of aspirin. The animals were killed by stunning and cervical dislocation 3 h after the administration of aspirin and the stomach was removed. Gastric mucosal lesions were measured by two independent observers who were blinded to the treatment. The ulcer index was calculated as the sum of the lengths of all lesions^[22].

Effect of ATL-146e on myeloperoxidase in gastric mucosa

Gastric mucosal myeloperoxidase (MPO) concentration

was assayed to quantify the degree of neutrophil infiltration. Three hundred milligrams of scraped mucosa was homogenized for 30 s with a polytron homogenizer (PT 1200, Kinematica AG, Littau, Switzerland) in 1.0 mL of ice-cold 5g/L hexadecyltrimethylammonium bromide in 50 mmol/L phosphate buffer (pH 6.0). Hexadecyltrimethylammonium bromide was used to negate the pseudoperoxidase activity of hemoglobin and to solubilize membrane-bound MPO. The homogenate was sonicated (U50 IKA Werke GmbH and Co. KG, Staufen, Germany) for 10 s, freeze-thawed thrice and centrifuged at 18000 *g* for 20 min. The supernatant was taken for the determination of the enzyme activity utilizing an ELISA kit (Bioxytech, Oxis International, Inc., Portland, OR, USA). The change in absorbance at 405 nm was measured with a spectrophotometer (Microplate reader model 3550, Bio-Rad, Hercules, CA, USA). The concentration of MPO was expressed as nanogram per milligram protein measured using Bradford's method^[23].

Effect of ATL-146e on gastric of TNF- α and IL-1 β

One hundred milligrams of scraped mucosa was homogenized for 30 s with a polytron homogenizer (PT 1200, Kinematica AG, Littau, Switzerland) in 1.0 mL of ice-cold potassium phosphate buffer (pH 7.4). Aliquots of homogenate supernatants in PBS were obtained by centrifugation at 10000 *g* for 10 min. Total protein was measured by Bradford's method. Concentration of TNF- α and IL-1 β in the supernatant of mucosal homogenates was determined by ELISA (R&D Systems Inc., Minneapolis, MN, USA) according to the manufacturer's instructions. After color development, optimal density was measured with a microplate reader. The concentration of TNF- α and IL-1 β was expressed as pictogram per milligram protein.

Effect of ATL-146e on gastric secretion

Gastric juice was collected using the pylorus ligation method^[24]. Briefly, rats were fasted for 24 h, placed in restraint cages, and injected intraperitoneally with 5 μ g/kg of ATL-146e. Thirty minutes after the injection, pylorus ligation was performed. The stomach of the rats were removed 3 h after pylorus ligation, and the gastric contents were collected. Following centrifugation, acid content in the supernatant determined by titration with 0.01 mol NaOH to pH 7.0 using a pH meter (Φ 50 pH Meter; Beckman, Tokyo, Japan), was used to calculate gastric acid secretion in mmol/3 h.

Effect of ATL-146e on mucosal PGE₂

To evaluate the effect of ATL-146e on mucosal content of PGE₂, rats were divided into four groups. In group A, rats were treated with vehicle. In group B, rats were treated with 5 μ g/kg of ATL-146e. Thirty minutes later, the animals were killed. In group C, vehicle was injected intraperitoneally 30 min prior to the administration of aspirin and were killed 3 h later. In group D, ATL-146e (5 μ g/kg) was injected intraperitoneally 30 min prior to the administration of aspirin and killed 3 h later. A part of fundic mucosa (about 100 mg) was excised for the

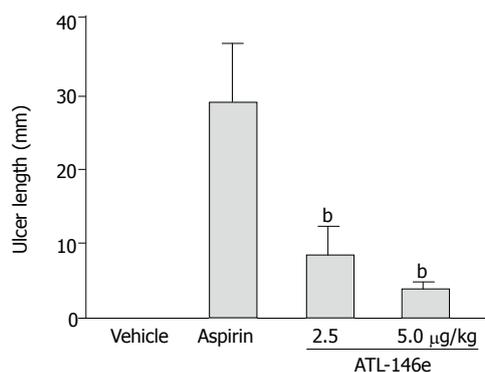


Figure 1 Ulcer length of rats 3 h after the administration of aspirin with or without pretreatment with ATL-146e (2.5 or 5 µg/kg, ip) ($n=5$ each group). $^bP<0.01$ vs aspirin alone treated group.

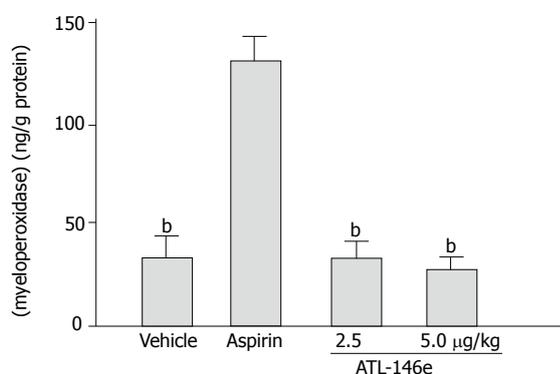


Figure 2 Effect of ATL-146e on myeloperoxidase concentration in gastric mucosa ($n=5$ each group). $^bP<0.001$ vs aspirin alone treated group.

determination of PGE₂ synthesis. The samples were weighed, finely minced with scissors for 15 s, and then suspended in 1.0 mL of 10 mmol/L sodium phosphate buffer (pH 7.4). The samples were then incubated in a shaking bath (37 °C) for 29 min followed by centrifugation at 9 000 *g* for 30 s. The supernatant was frozen and subsequent determination of PGE₂ was performed by radioimmunoassay using PGE₂ [125I] RIA kit (Dupont/NEN, Boston, MA, USA).

Statistical analysis

All data were expressed as mean ± SE. Statistical significance was determined by ANOVA using Statview-J 4.11 statistical program (Abacus Concepts, Berkeley, CA, USA). $P<0.05$ was considered statistically significant.

RESULTS

Effect of ATL-146e on aspirin-induced gastric lesions

Administration of aspirin resulted in the appearance of linear and dotted erosions in the gastric mucosa of vehicle-treated rats. In contrast, pre-treatment with ATL-146e resulted in smaller erosions. The total length of gastric erosions (ulcer index) in control rats was 29.8 ± 7.7 mm. The ulcer index in rats pretreated with ATL-146e was significantly suppressed to 7.0 ± 2.34 mm (2.5 µg/kg, $P<0.05$), or 3.1 ± 1.42 mm (5.0 µg/kg, $P<0.01$) (Figure 1).

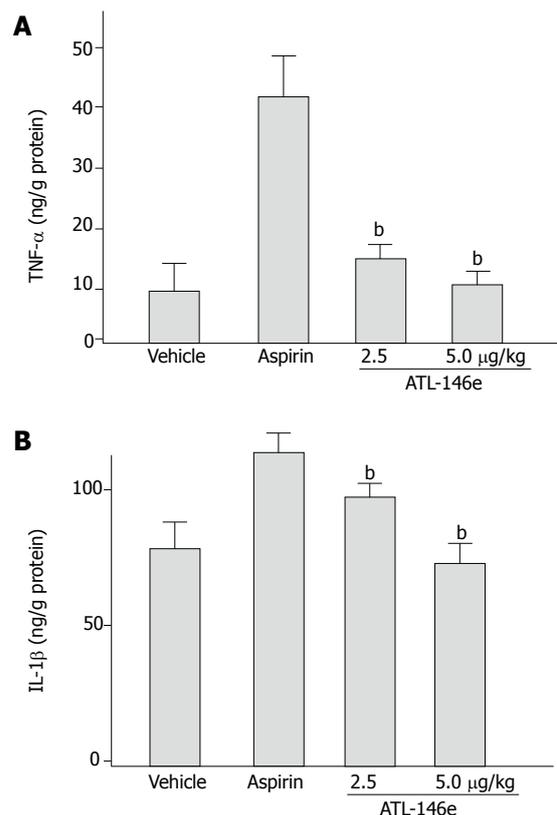


Figure 3 Effect of ATL-146e on increased TNF- α (A) and IL-1 β (B) concentration in gastric mucosa induced by aspirin administration ($n=5$ each group). $^bP<0.01$, vs aspirin alone treated group.

Effect of ATL-146e on MPO in gastric mucosa

Tissue MPO concentration in the gastric mucosa increased 3 h after the initiation of administration of aspirin. The MPO concentration in normal control animals (2.8 ± 0.2 µg/g protein) increased to 13.8 ± 1.2 µg/g protein in vehicle-treated rats. The increment of MPO concentration in the gastric mucosa by aspirin was suppressed by pretreatment with ATL-146e to 2.9 ± 0.35 µg/g protein (2.5 µg/kg, $P<0.001$) or 2.7 ± 0.14 µg/g protein (5 µg/kg, $P<0.001$) compared to that in vehicle-treated rats (Figure 2). The increase in MPO above control levels caused by aspirin was reduced to nearly normal levels after the administration of 2.5 and 5 µg/kg ATL-146e.

Effect of ATL-146e on gastric of TNF- α and IL-1 β

The gastric concentrations of TNF- α and IL-1 β were significantly increased 3 h after the administration of aspirin. ATL-146e at the doses of 2.5 and 5.0 µg/kg significantly suppressed the increment of tissue TNF- α and IL-1 β in the gastric mucosa by the administration of aspirin (Figure 3). The increase in TNF- α above the control levels caused by aspirin was reduced by the administration of 2.5 and 5 µg/kg ATL-146e by 90.3% and 99.7%, respectively. The increase in IL-1 β above the control levels caused by aspirin was reduced by the administration of 2.5 and 5 µg/kg ATL-146e by 64.0% and 97.2%, respectively.

Effect of ATL-146e on gastric secretion

ATL-146e caused a three-fold increase in gastric acid output from 114 to 326 mmol/3h ($P<0.05$, Figure 4).

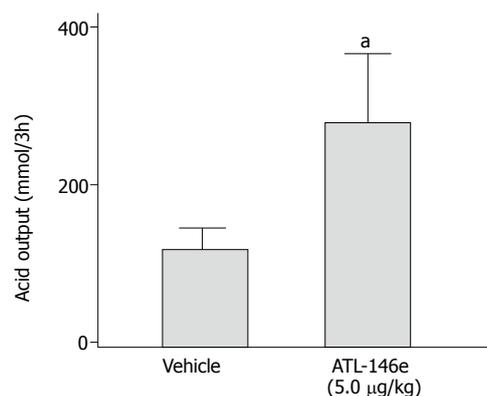


Figure 4 Effect of 5 µg/kg ATL-146e on gastric acid secretion. Rats were treated with or without 5.0 µg/kg of ATL-146e and killed 3 h later ($n=5$ each group). ^a $P<0.05$ vs vehicle treated group.

Effect of ATL-146e on mucosal of PGE₂

The concentration of PGE₂ was 255.3 ± 64.7 ng/g in vehicle-treated rats (group A) and 47.4 ± 5.6 ng/g in aspirin-treated animals (group C) (82.3% reduction, $P<0.05$). ATL-146e (5 µg/kg) administration did not interfere with the reduction of gastric PGE₂ concentration induced by aspirin ($P>0.05$ vs aspirin alone) (Figure 5).

DISCUSSION

The results of this study clearly showed that a single bolus injection of the adenosine A_{2A} agonist, ATL-146e 30 min prior to the administration of aspirin, could effectively reduce the extent of gastric mucosal lesions. This protection is correlated with the inhibition of neutrophil infiltration into the gastric mucosal tissue and production of pro-inflammatory cytokines in gastric mucosal tissue. Previous studies have shown that gastric mucosal MPO concentration, a biochemical indicator of neutrophils, increases with the development of gastric mucosal lesions^[25]. These findings suggest that most of the stimuli for aspirin-induced gastric injury that is susceptible to the inhibition by ATL-146e may occur early after the administration of aspirin.

Recent studies reported that gastric mucosal lesions can be reduced by the administration of antibodies against TNF- α ^[26]. Therefore, we may postulate that infiltration of neutrophils in rat gastric mucosa after the administration of aspirin could occur in response to the gastric production of pro-inflammatory cytokines, resulting in the development of gastric mucosal lesions secondary to neutrophil accumulation. Anti-inflammatory and tissue protective effects of ATL-146e in the models of ischemia-reperfusion injury have been reported^[13,14,27-29].

Adenosine has been identified as an endogenous anti-inflammatory agent because the activation of the A_{2A} receptor is known to increase intracellular cAMP levels and to reduce diverse leukocyte functions^[30]. Ross *et al.*^[14] demonstrated that ATL-146e protects lung from reperfusion injury by reducing neutrophil sequestration^[14]. Our recent study reported that ATL-146e inhibits water-immersion stress-induced gastric injury due to the inhibition of neutrophil accumulation and reduction of

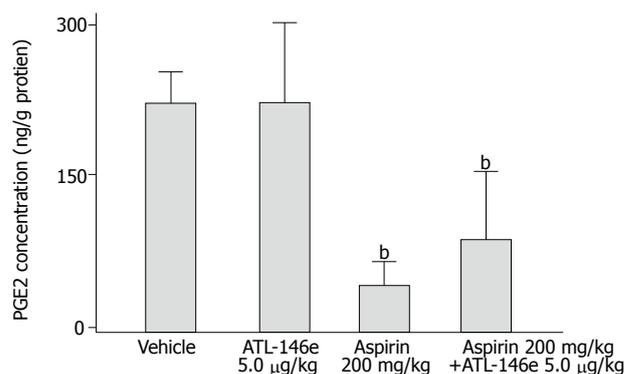


Figure 5 Gastric mucosal concentrations of prostaglandin E₂ in control group (vehicle group) and in rats treated with 200 mg/kg aspirin alone or pretreated with 5 µg/kg ATL-146e ($n=5$ each group). ^b $P<0.01$ vs vehicle treated group. NS: no significant difference between each group.

pro-inflammatory cytokine production^[19]. These findings led us to examine the effect of ATL-146e on aspirin-induced gastric mucosal lesion. In the present study, we have demonstrated that MPO concentration, an index of tissue-associated neutrophil accumulation, increased in the gastric mucosa 3 h after the administration of aspirin. The increased MPO concentration was significantly inhibited by treatment with ATL-146e.

TNF- α is a pro-inflammatory cytokine and has recently been shown to be a crucial mediator of NSAIDs-induced gastric mucosal injury^[31]. Also, TNF- α is a cytokine that strongly stimulates neutrophil adherence by inducing synthesis and expression of adhesion molecules on endothelial cells and neutrophils^[32,33]. TNF- α augments neutrophil-derived superoxide generation and upregulates the expression of adhesion molecules on neutrophil and endothelium, and stimulates production of IL-1 β , leading to neutrophil accumulation^[32,33]. Furthermore, studies on experimental models have shown that intravenous administration of TNF- α produces extensive neutrophil infiltration within the microvasculature of the digestive tract. In addition, portal infusion of TNF- α causes gastric and small intestinal damage in rats^[34]. The present study demonstrated that ATL-146e treatment could inhibit increase of TNF- α and IL-1 β concentration in the gastric mucosa after the administration of aspirin. These findings strongly support the hypothesis that ATL-146e attenuates aspirin-induced neutrophil accumulation by inhibiting production of pro-inflammatory cytokines.

Okusa *et al.*^[13] reported that ATL-146e inhibits ischemic reperfusion injury of kidney not only by reducing neutrophil accumulation, but also by reducing the expression of the adhesion molecules, P-selectin, and ICAM-1 on the reperfused vascular endothelium. Andrews *et al.*^[6] also reported that the expression of ICAM-1 on endothelial cells is increased by NSAIDs. Therefore, the anti-ulcer effect of ATL-146e might be due to the inhibition of neutrophil adhesion.

In addition, it is well established that various anti-secretory agents, such as H₂-receptor antagonists and proton pump inhibitors prevent gastric lesions^[35]. However, we found that ATL-146e could significantly increase gastric acid secretion. These data indicate that

the protective effect of ATL-146e is not due to reduced gastric acid secretion. Also, it has been reported that PGE₂ prevents gastric mucosal damage by aspirin in human beings and animals^[36]. The protective effect of ATL-146e is not dependent on gastric mucosal prostaglandin synthesis, since pretreatment with ATL-146e, which reduces gastric damage, has no effect on the gastric mucosal prostaglandin concentration. Therefore, inhibition of mucosal lesions by ATL-146e cannot be attributed to gastric acid inhibition and prostaglandin synthesis.

In conclusion, the potent and selective adenosine A_{2A} receptor agonist, ATL-146e, significantly inhibits acute gastric mucosal injury induced by aspirin in rats. This effect may be due in part to a reduction in neutrophil infiltration into the gastric mucosa and inhibition of pro-inflammatory cytokines production. Also, modulation of adenosine A_{2A} receptor activity by specific agonists may be clinically useful for the therapy of NSAIDs-induced gastric mucosal damage.

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BASIC RESEARCH

Role of gastric oxidative stress and nitric oxide in formation of hemorrhagic erosion in rats with ischemic brain

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Abstract

AIM: To investigate the role of gastric oxidative stress and nitric oxide (NO) in the formation of gastric hemorrhagic erosion and their protection by drugs in rats with ischemic brain.

METHODS: Male Wistar rats were deprived of food for 24 h. Under chloral hydrate (300 mg/kg) anesthesia, bilateral carotid artery ligation was performed. The pylorus and carotid esophagus of the rats were also ligated. The stomachs were then irrigated for 3 h with either normal saline or simulated gastric juice containing 100 mmol/L HCl plus 17.4 mmol/L pepsin and 54 mmol/L NaCl. Rats were killed and stomachs were dissected. Gastric mucosa and gastric contents were harvested. The rat brain was dissected for the examination of ischemia by triphenyltetrazolium chloride staining method. Changes in gastric ulcerogenic parameters, such as decreased mucosal glutathione level as well as enhanced gastric acid back-diffusion, mucosal lipid peroxide generation, histamine concentration, luminal hemoglobin content and mucosal erosion in gastric samples, were measured.

RESULTS: Bilateral carotid artery ligation produced severe brain ischemia (BI) in rats. An exacerbation of various ulcerogenic parameters and mucosal hemorrhagic erosions were observed in these rats. The exacerbated ulcerogenic parameters were significantly ($P < 0.05$) attenuated by antioxidants, such as exogenous glutathione and allopurinol. These gastric parameters were also improved by intraperitoneal aminoguanidine (100 mg/kg) but were aggravated by *N*^ε-nitro-L-arginine-methyl ester (L-NAME: 25 mg/kg). Intraperitoneal L-arginine (0-500 mg/kg) dose-dependently attenuated BI-induced aggravation of ulcerogenic parameters and hemorrhagic erosions that were reversed by L-NAME.

CONCLUSION: BI could produce hemorrhagic erosions

through gastric oxidative stress and activation of arginine-nitric oxide pathway.

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Key words: Lipid peroxide; Acid back-diffusion; Glutathione; Allopurinol; L-arginine; Nitric oxide synthase; Aminoguanidine

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INTRODUCTION

Brain ischemia (BI) resulting from cardiovascular stenosis, hypercholesterolemia or intracranial hemorrhage is frequently found in the clinic. The development of this disease may lead to cerebral hypoxia, brain tissue damage, and stroke. The peripheral manifestations of BI may include seizures^[12], neurological deficiency^[15] and hemostatic disturbance^[8]. However, whether gastric mucosal integrity is also impaired during the occurrence of BI is still unknown till now. In general, the disruption and/or degeneration of gastric mucosal cells may result in the decrease in gastric defensive factors, including mucosal reduced glutathione (GSH), and/or in increase of offensive factors, such as gastric acid back-diffusion and generation of oxyradicals. Our previous papers indicated that acid back-diffusion was a critical factor of acid-induced exacerbation of gastric hemorrhagic ulcer in diabetic or starved rats^[19,20]. The back-diffused free acid may damage gastric mucosal cells by increasing oxyradicals and the release of histamine^[15]. In fact, oxyradicals produced during oxidative stress play a pivotal role in the etiology of many diseases^[26], including cancer^[1] and sepsis^[14,15]. However, effects of gastric oxidative stress and oxyradical scavengers, such as exogenous reduced GSH or allopurinol on the formation of gastric hemorrhage and mucosal erosion in gastric juice-irrigated stomachs of rats with ischemic brain, remain obscure.

Ample documents demonstrate that nitric oxide (NO) plays an important role in physiological vasodilatation, cytotoxicity and vascular diseases. NO and prostacyclin released from the endothelium may act synergistically to inhibit platelet aggregation and adhesion^[36] that are

associated with pathophysiological mechanisms of many diseases^[10]. L-arginine, an NO donor, is an important amino acid nutrient in living organs. Whether this amino acid can protect gastric mucosa against gastric hemorrhage and stomach erosion through the production of NO in rats with ischemic brain is also unknown. In the present study, the non-selective NO synthase inhibitor, N^G-nitro-L-arginine-methyl ester (L-NAME) or relatively selective inducible NO synthase (iNOS) inhibitor, aminoguanidine (AMG), were used to clarify the role of NO in the protection of gastric mucosa against damage in rats with ischemic brain. Taken together, the aim of the present study was to investigate whether or not oxidative stress-induced changes in acid back-diffusion, histamine release and mucosal oxyradical generation as well as in GSH level and NO liberation are important to modulate gastric hemorrhagic erosion in stomachs of rats with ischemic brain.

MATERIALS AND METHODS

Animals

Male Wistar rats, weighing 200-250 g, were obtained from and housed in the Laboratory Animal Center, National Cheng Kung University, Tainan, Taiwan. Rats were housed individually in a room with 12-h dark-light cycle and central air conditioning (25 °C temperature, 70% humidity). Rats were allowed free access to water and pellet diets (the Richmond standard, PMI Feeds, Inc., St. Louis, MO, USA). The animal care and experimental protocols were in accordance with the guidelines of the National Science Council of Taiwan (NSC 1994). Before the experiment, rats were deprived of food for 24 h. BI induced by bilateral carotid artery ligation (BCAL) for 3 h was carried out under potent anesthesia of intraperitoneal chloral hydrate (300 mg/kg). Adequate amount of chloral hydrate was injected to rats for the maintenance of anesthesia. To maintain spontaneous respiration, a polypropylene tube (3.0 mm in diameter and 50 mm in length) was intubated in the rat trachea. Control rats received sham BCAL. At the end of the experiment, rat brains were dissected and sliced for 2-mm thick at a distance of 4, 6, and 8 mm from the forebrain. Brain slices were soaked in triphenyltetrazolium chloride solution for 30 min at 37 °C as described by Isayama *et al*^[18]

Chemicals

The following reagent-grade chemicals were used. Acivicin, allopurinol, AMG, L-arginine, chloral hydrate, *n*-butanol, 2,2'-dinitro-5,5'-dithio-dibenzoic acid, pyridine, L-NAME, *o*-phthaldialdehyde (OPT), rat hemoglobin, reduced GSH, sodium lauryl sulfate, 1,1,3,3-tetramethoxypropane, trichloroacetic acid and 2-thiobarbiturate were purchased from Sigma, St. Louis, MO, USA. The purity of all drugs was over 98%. All chemical solutions were freshly prepared before use.

Drug administration

Intraperitoneal L-arginine (0-500 mg/kg), AMG (100 mg/kg), L-NAME (25 mg/kg, i.p.), GSH (800 mg/kg) or allopurinol (100 mg/kg) were administered to rats 30 min before gastric juice irrigation.

Gastric surgical procedures

Rat stomachs were surgically exposed for the ligation of pylorus and carotid esophagus. A small incision was made in the forestomach of rats. The stomach contents were gently expelled from the incision. A polypropylene tube (1.0 mm internal diameter×20 mm long) was inserted through the same incision and secured with a ligature. Subsequently, the stomach was rinsed meticulously with warm saline (37 °C). Care was taken to avoid gastric distension. The residues were gently removed.

Measurement of gastric acid back-diffusion

Gastric acid back-diffusion (luminal H⁺ loss) was quantified by the method as previously described^[14]. Briefly, 7-mL of either normal saline or simulated gastric juice containing 100 mmol/L HCl, 17.4 mmol/L pepsin and 54 mmol/L NaCl^[25] were instilled into the cleansed stomach with a syringe. The luminal contents were mixed with the same syringe by three repeated aspirations and injection, and 3 mL of the fluid was taken as an initial sample. The forestomach was tightly closed. The abdominal wound was sutured. After 3 h, rats were killed with an overdose of ether. The gastric sample (final sample) was collected and centrifuged for 20 min at 3 000 r/min

Quantitation of gastric sample

The volumes of the initial and final samples were measured. Gastric acidity of the samples was assessed by titrating 1.0 mL of sample gastric contents with 0.1 mol/L NaOH to pH 7.0 on an autoburette titrator (Radiometer, Copenhagen, Denmark). The net flux of ions through gastric mucosa was calculated as follows: Net flux = $F_v \times F_c - (7 - I_v) \times I_c$, where F_v and I_v are the volumes (mL) of final sample and initial sample, respectively, while F_c and I_c are the ionic concentrations (mmol/L) in the final sample and initial sample, respectively. The negative value means the luminal electrolyte loss and the positive value indicates the luminal electrolyte gain.

Morphological and histological studies of gastric mucosa

As soon as the final sample was collected, the stomach was filled with 10 mL/L formalin for 10 min. The mucosa was exposed by opening the stomach along the greater curvature. The length (mm) and the width (mm) of erosion on the gastric mucosa were measured with planimeter (1 mm×1 mm) under a dissecting microscope (×0.7-×3.0; American Optical Scientific Instrument 569, Buffalo, NY, USA). The erosion areas were determined as previously described^[14]: Erosion area = length×width× $\pi/4$. The total erosion area (mm²) of each stomach was recorded. Histological studies of the stomach were also conducted by methods as previously described^[6]. Briefly, after gross examination, the specimens taken from the stomachs were blocked and immersed into 100 mL/L neutral formalin for 2 d. Blocks were then dehydrated in series of alcohol, cleared in xylene and embedded in paraffin. Sections (7 μ m thickness) were cut and stained with hematoxylin and eosin as routine histological procedures. Each section was examined under a microscope (Nikon HF, X-IIA, Tokyo, Japan), and the tissue damage was quantified. Sections were scored as 0-5, in which 0 indicated a normal

appearance, 1 indicated mild injury in the epithelial cells, 2 indicated mild injury in the upper part of mucosal cells, 3 indicated hemorrhage or edema in the mid or lower part of mucosal cells, 4 indicated degranulation or necrosis of the epithelial cells and 5 indicated serious cell disruption of lower part of the mucosa. The index score of each section was evaluated on a cumulated basis to give a maximal score of 15.

Determination of hemoglobin

The cleansed rat stomachs were irrigated for 3 h with either saline or simulated gastric juice. Initial and final gastric samples were collected by aforementioned methods. The blood attached on the gastric mucosa was carefully scraped and added into the final sample. Subsequently, both initial and final samples were adjusted to pH 1.5 with 0.1 mol/L HCl. The concentrations of hemoglobin in samples were measured spectrophotometrically^[15]. The absorption maximum of Hb was measured at 376 nm. The appropriate irrigated solutions adjusted to pH 1.5 were used as blank. Absorbances of samples were measured against a standard curve ($r > 0.90$) contrasted with freshly prepared rat Hb (0.05-1.00 g/L) treated in the same manner as gastric samples. The luminal Hb content was calculated as $F_v \times F_{Hb} - (7 - I_v) \times I_{Hb}$, where F_v and I_v are the volumes (mL) of the final sample and initial sample, respectively, while F_{Hb} and I_{Hb} are the luminal Hb concentrations (g/L) in the final sample and initial sample, respectively. The results obtained from gastric samples were expressed as milligram Hb per stomach.

Assay of mucosal GSH

The quantitation of gastric mucosal GSH was performed by methods as previously demonstrated^[19]. After the final sample was collected, the rat stomach was dissected. The corpus mucosa was scraped using two glass slides on ice, weighed and homogenized immediately in 2 mL of phosphate buffer (0.1 mol/L NaH_2PO_4 plus 0.25 mol/L sucrose, pH 7.4). Acivicin (250 $\mu\text{mol/L}$), an irreversible inhibitor of γ -glutamyltransferase, was added to the homogenate to inhibit the catabolism of GSH. The samples were then centrifuged at 4000 r/min for 15 min at 4 °C. To determine the recovery of reduced thiol, the supernatant was added with or without GSH (200 μmol of reduced GSH contained in phosphate buffer solution, pH 7.0). Subsequently, 0.5 mL of 0.25 mol/L trichloroacetic acid was added to 1.0 mL of the supernatant of each sample and kept for 30 min at 4 °C. After centrifugation for 15 min at 3 000 r/min, the supernatant was used to determine GSH using 2,2'-dinitro-5,5'-dithio-dibenzoic acid. The optical density was measured at 412 nm on a Hitachi spectrophotometer (model U-3210, Tokyo, Japan). All samples were measured in duplicate. Recovery of added internal standard was greater than 90% in all experiments. Absorbances of the samples were measured against a standard curve constructed with freshly prepared GSH solutions (0.05-0.5 mmol/L), which were treated in the same manner as the tissue samples. The results obtained from tissue samples were expressed as micromole per gram wet tissue.

Determination of mucosal lipid peroxides

The concentrations of gastric mucosal lipid peroxides (LPO) were determined by estimating malondialdehyde (MDA) using thiobarbituric acid test^[32]. Briefly, the stomachs of rats were promptly excised and rinsed with cold saline. To minimize the possibility of interference of Hb with free radicals, any blood adhering to the mucosa was carefully removed. The corpus mucosa was scraped, weighed and homogenized in 10 mL of 100 g/L KCl. The homogenate (0.5 mL) was added with a solution containing 0.2 mL of 80 g/L sodium laurylsulfate, 1.5 mL of 200 g/L acetic acid, 1.5 mL of 8 g/L 2-thiobarbiturate and 0.3 mL of distilled water. The mixture was incubated at 98 °C for 1 h. Upon cooling, 5 mL of n-butanol:pyridine (15:1) was added. The mixture was vortexed for 1 min and centrifuged for 10 min at 4000 r/min. The supernatant was measured at 532 nm. The standard curve was obtained by using 1,1,3,3-tetramethoxypropane. The recovery was over 90%. All samples were measured in duplicate. The results were expressed as nanomole MDA per gram wet tissue.

Measurement of mucosal histamine

Gastric mucosal histamine concentration was determined by the methods as described previously^[33]. Briefly, gastric mucosa was scraped and homogenized with trichloroacetic acid (90 mmol/L) in a final concentration of 100 g tissue/L. The homogenate was further centrifuged at 54000 r/min for 10 min. The OPT-NaOH solution was prepared by dissolving 10 mg of OPT in 1.0 mL methanol (10 g/L) and 4 mL of NaOH (0.06 mol/L). This mixed solution was then gassed with nitrogen for 10 min. Then, 2.2 mL of a 20 g/L OPT-NaOH solution was added to 100 μL of a 1/10-fold diluted sample of supernatant or histamine test solution. The mixture was placed at -20 °C for 10 h. Then 200 μL of 0.35 mol/L H_2SO_4 (final pH 1.6-2.4) was added to this frozen mixture. After thawing, the sample was vortexed for 1 min. The fluorescence of the sample was read at room temperature (25 °C) at 350 and 450 nm on a fluorescent spectrophotometer (Model 251-0030, Tokyo, Japan) using 1 cm^2 quartz cells. All samples were measured in duplicate. The fluorescence of the sample was calculated against a standard curve constructed with freshly prepared histamine solutions (0.78-25 mg/L) that were treated in the same manner as the tissue samples. The results obtained from tissue samples were expressed as microgram histamine per gram wet tissue.

Statistical analysis

The data obtained from the experiments were expressed as mean \pm SE. Differences in the data of experiments were analyzed statistically using ANOVA^[28]. A simple regression analysis was used to determine the correlation between two different variances. $P < 0.05$ was considered statistically significant.

RESULTS

Morphological changes of gastric mucosa of rats with ischemic brain

Gastric mucosa was morphologically intact in sham

Table 1 Influences of gastric juice on various gastric biomedical parameters in sham operation and BCAL rats

| | Acid back-diffusion ($\mu\text{Eq}/\text{stomach}$) | Glutathione ($\mu\text{mol}/\text{g}$ tissue) | Lipid peroxide (nmol MDA/g tissue) | Hemoglobin (mg/ stomach) | Erosion (area mm^2) | Histological score |
|----------------|--|--|--|--------------------------------|-------------------------------------|----------------------------|
| Sham operation | | | | | | |
| Saline | 56.0 \pm 6.0 | 3.0 \pm 0.4 | 36.1 \pm 4.5 | 0.1 \pm 0.1 | 0.3 \pm 0.2 | 0.3 \pm 0.1 |
| Gastric juice | -135.6 \pm 4.4 | 2.6 \pm 0.3 | 50.1 \pm 6.4 | 0.3 \pm 0.1 | 0.4 \pm 0.8 | 0.7 \pm 0.3 |
| BCAL | | | | | | |
| Saline | 30.2 \pm 4.1 ^a | 2.1 \pm 0.3 ^a | 88.1 \pm 3.4 ^a | 0.8 \pm 0.2 ^a | 8.2 \pm 0.9 ^a | 1.8 \pm 0.3 ^a |
| Gastric juice | -250.2 \pm 10.4 ^c | 1.4 \pm 0.2 ^c | 137.6 \pm 10.4 ^c | 2.0 \pm 0.4 ^c | 40.2 \pm 7.1 ^c | 4.5 \pm 0.7 ^c |

Data are means \pm SE (N=8). Significant differences are analyzed by using ANOVA. ^a $P < 0.05$ vs saline treated sham operation group; ^c $P < 0.05$ vs gastric juice treated sham operation group, BCAL= bilateral carotid artery ligation, MDA = malondialdehyde.

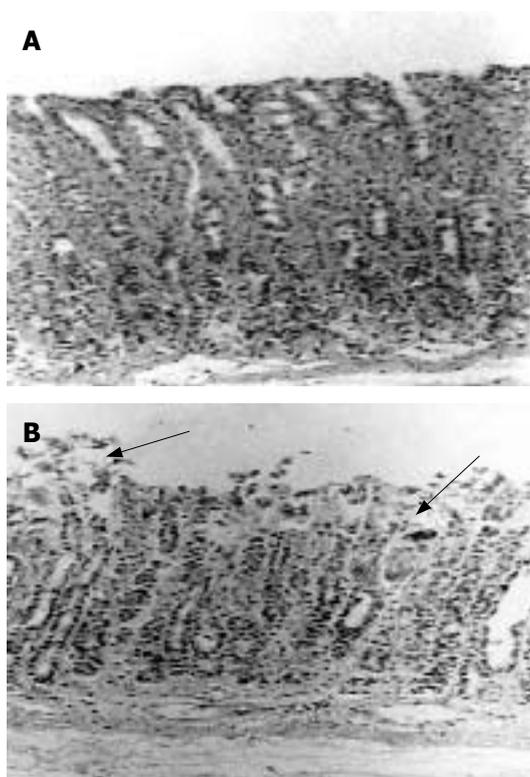


Figure 1 Histological study of gastric mucosa in sham operative rats and rats with ischemic brain. **A:** In sham operative rat stomachs irrigated with gastric juice, gastric mucosal cells appear intact; **B:** Disruption of the upper mucosal cells and lamina propria (indicated by arrows) in rats with ischemic brain, the injured cells are characterized by karyorrhexis and dense homogenous acidophilic cytoplasm (HE \times 150)

operative rats. Nevertheless, gastric hemorrhagic erosions were observed in the stomachs of rats with ischemic brain (photo not shown). Histological studies showed that gastric mucosal cells appeared intact in sham operative rat stomachs irrigated with gastric juice (Figure 1A). However, necrotic cell-injury was found in both epithelial layers and lamina propria when gastric juice was present in the stomachs of rats with ischemic brain (Figure 1B). In sham operative rat stomachs irrigated with normal saline, no damage of gastric mucosal cells was observed. In gastric juice-irrigated stomachs of sham operative rats, gastric mucosal cells also appeared undamaged. In rats with ischemic brain, normal saline-irrigated stomachs produced a little gastric mucosal cell damage than did

those treatments in sham operative rats. Furthermore, a pronounced aggravation of mucosal cell damage was observed when gastric juice was used instead of normal saline in these rats. Apparently, intra-luminal gastric juice could enhance mucosal cell damage in rats with ischemic brain (Table 1).

We observed that back-diffused H^+ concentrations, luminal Hb contents and mucosal erosion in normal saline-irrigated stomachs of sham operative rats were negligible (Table 1). Gastric mucosal GSH concentrations and LPO generations in these rats were also at normal levels. Similar results were observed in gastric juice-irrigated stomachs of sham operative rats. In rats with ischemic brain, normal saline-irrigated stomachs produced significant increases in these gastric parameters compared with those found in sham operative rat stomachs irrigated with normal saline ($P < 0.05$). When gastric juice was used instead of normal saline in rats with ischemic brain, remarkable exacerbations of gastric hemorrhagic erosions accompanied with great enhancement in acid back-diffusion and mucosal LPO as well as a lowering of mucosal GSH levels were observed. High correlations between exacerbated gastric mucosal damage and decreased GSH levels as well as between mucosal erosions and increased LPO were observed in those gastric juice-irrigated stomachs of rats with ischemic brain (Figure 2). Apparently, BI could produce gastric oxidative stress in rats. Furthermore, gastric juice was able to aggravate various ulcerogenic parameters in rats with ischemic brain.

Effect of allopurinol or exogenous GSH

Intraperitoneal allopurinol (100 mg/kg) or reduced GSH (800 mg/kg) caused a significant decrease in acid back-diffusion and LPO generation as well as histamine concentration and hemorrhagic erosion in sham operative rats ($P < 0.05$, Table 2). Gastric mucosal GSH levels were elevated in exogenous GSH-treated rats. In rats with ischemic brain, these two chemicals produced a pronounced inhibition in acid back-diffusion, LPO generation, histamine release and hemorrhagic erosions while GSH levels were greatly elevated.

Effect of L-arginine

We observed that L-arginine (500 mg/kg) significantly increased gastric mucosal GSH levels but decreased acid back-diffusion, LPO and histamine concentrations in gastric juice-irrigated stomachs of sham operative rats

Table 2 Effects of allopurinol or exogenous glutathione on various gastric parameters in sham operation and BCAL rats

| | mg/kg | Acid back-diffusion ($\mu\text{Eq}/\text{stomach}$) | Glutathione ($\mu\text{mol}/\text{g tissue}$) | Lipid peroxide (nmol MDA/g tissue) | Histamine ($\mu\text{g}/\text{g tissue}$) | Hemoglobin (mg/stomach) | Erosion area (mm^2) |
|----------------|-------|--|--|---------------------------------------|--|----------------------------|-----------------------------------|
| Sham operation | | | | | | | |
| Vehicle | | -135.4 \pm 4.4 | 2.8 \pm 0.3 | 50.1 \pm 4.5 | 56.1 \pm 5.4 | 0.5 \pm 0.2 | 2.4 \pm 3.5 |
| Allopurinol | 100 | -112.0 \pm 3.8 ^a | 3.0 \pm 0.3 | 40.2 \pm 4.7 ^a | 40.5 \pm 5.1 ^a | 0.2 \pm 0.1 ^a | 0.2 \pm 0.2 ^a |
| Glutathione | 800 | -98.0 \pm 2.4 ^a | 3.4 \pm 0.2 ^a | 36.1 \pm 2.5 ^a | 38.1 \pm 4.6 ^a | 0.1 \pm 0.1 ^a | 0.4 \pm 0.2 ^a |
| BCAL | | | | | | | |
| Vehicle | | -250.2 \pm 14.4 | 1.4 \pm 0.2 | 128.0 \pm 9.3 | 120.4 \pm 9.8 | 2.3 \pm 0.3 | 45.2 \pm 6.1 |
| Allopurinol | 100 | -181.5 \pm 5.8 ^c | 2.2 \pm 0.2 ^c | 68.4 \pm 5.1 ^c | 77.3 \pm 6.9 ^c | 1.2 \pm 0.2 ^c | 20.3 \pm 2.9 ^c |
| Glutathione | 800 | -146.0 \pm 3.4 ^c | 2.7 \pm 0.2 ^c | 56.1 \pm 4.5 ^c | 80.1 \pm 4.6 ^c | 0.1 \pm 0.1 ^c | 12.4 \pm 3.5 ^c |

Data are means \pm SE (N=6). ^a*P*< 0.05 vs sham operative vehicle. ^c*P*< 0.05 vs BCAL vehicle. MDA= malonedialdehyde, BCAL= bilateral carotid artery ligation.

Table 3 Effect of L-NAME on L-arginine produced amelioration of various gastric biomedical parameters in sham operation and BCAL rats

| | mg/kg | Acid back-diffusion ($\mu\text{Eq}/\text{stomach}$) | Glutathione ($\mu\text{mol}/\text{g tissue}$) | Lipid peroxides (nmol MDA/g tissue) | Histamine ($\mu\text{g}/\text{g stomach}$) | Hemoglobin (mg/stomach) | Erosion area (mm^2) |
|--------------------------|-------|--|--|--|---|-----------------------------|-----------------------------------|
| Sham operation | | | | | | | |
| Vehicle | | -125.2 \pm 4.4 | 2.6 \pm 0.3 | 48.1 \pm 3.4 | 52.8 \pm 4.6 | 0.1 \pm 0.1 | 3.2 \pm 0.7 |
| L-arginine | 500 | -96.0 \pm 6.0 ^a | 3.5 \pm 0.4 ^a | 36.1 \pm 4.5 ^a | 38.2 \pm 3.6 ^a | 0 \pm 0 ^a | 0.3 \pm 0.2 ^a |
| L-NAME25 + L-arginine | 500 | -156.0 \pm 11.0 ^{ac} | 2.3 \pm 0.2 ^c | 60.1 \pm 4.5 ^{ac} | 72.4 \pm 6.3 ^{ac} | 0.7 \pm 0.1 ^{ac} | 12.0 \pm 3.5 ^{ac} |
| BCAL | | | | | | | |
| Vehicle | | -245.2 \pm 14.4 | 1.6 \pm 0.2 | 127.8 \pm 9.3 | 110.4 \pm 9.8 | 1.6 \pm 0.3 | 36.2 \pm 5.1 |
| L-arginine | | | | | | | |
| | 25 | -216.1 \pm 9.6 ^a | 2.0 \pm 0.2 | 80.8 \pm 7.8 ^a | 98.7 \pm 10.4 | 1.3 \pm 0.2 | 28.2 \pm 5.4 |
| | 250 | -189.4 \pm 3.6 ^a | 2.3 \pm 0.3 ^a | 66.7 \pm 4.4 ^a | 84.3 \pm 7.4 ^a | 1.0 \pm 0.1 ^a | 20.0 \pm 4.8 ^a |
| | 500 | -144.0 \pm 11.0 ^a | 2.6 \pm 0.2 ^a | 46.1 \pm 8.5 ^a | 54.1 \pm 8.6 ^a | 0.7 \pm 0.1 ^a | 12.0 \pm 3.5 ^a |
| L-NAME | 25 | | | | | | |
| + L-arginine | 500 | -287.6 \pm 16.4 ^{ac} | 1.2 \pm 0.2 ^{ac} | 157.6 \pm 11.3 ^{ac} | 110.4 \pm 9.8 ^{ac} | 2.8 \pm 0.3 ^{ac} | 56.2 \pm 6.0 ^{ac} |

Data are means \pm SE (N=6). ^a*P*<0.05 vs corresponding vehicle. ^c*P*<0.05 vs corresponding arginine treatment (500 mg/kg) of BCAL group. MDA= malonedialdehyde, BCAL= bilateral carotid artery ligation, L-NAME= NG-nitro-L-arginine-methyl ester.

(*P*<0.05, Table 3). In stomachs of rats with ischemic brain, the ulcerogenic parameters, such as increased acid back-diffusion, LPO generation, hemorrhage and mucosal erosions, were dose-dependently attenuated by pretreatment with L-arginine, whereas the decreased mucosal GSH levels found in these rats with ischemic brain were effectively inhibited. These cytoprotective effects of L-arginine on gastric mucosa were reversed by concomitant administration of L-NAME (25 mg/kg).

Effects of L-NAME or AMG

Intraperitoneal L-NAME caused significant aggravation of acid back-diffusion, LPO generation, histamine release and hemorrhagic erosions in rats with ischemic brain (*P*<0.05). Gastric mucosal GSH levels were attenuated (Figure 3). In AMG-treated rats with ischemic brain, a remarkable inhibition of acid back-diffusion, LPO generation and hemorrhagic erosion was achieved. Gastric mucosal GSH levels and histamine concentrations were significantly augmented (*P*<0.05).

DISCUSSION

The pathological mechanisms underlying the aggravation of gastric hemorrhagic erosion in gastric juice-irrigated stomachs of rats with ischemic brain are complex. The

present study demonstrated that the decreased GSH levels as well as increased mucosal LPO generation, histamine concentration and acid back-diffusion were exacerbated in rats with ischemic brain. We also observed the occurrence of gastric hemorrhage and erosion. Apparently, gastric oxidative stress was produced in rats with ischemic brain. During oxidative stress, gastric mucosal barriers were degenerated and disrupted. When gastric juice was irrigated in the stomachs of rats with ischemic brain, the intraluminal free H⁺ back diffused through disrupted barriers to the gastric mucosa and thereby damaged the gastric cells. Consequently, aggravation of gastric hemorrhagic erosion and various ulcerogenic parameters occurred. The back-diffused gastric acid may also stimulate oxyradical release. The elimination of gastric mucosal GSH levels in gastric juice-irrigated stomachs of rats with ischemic brain might be resulted from an increase in its consumption for scavenging oxyradicals, and from a decrease in its cellular biosynthesis. It is proposed that depletion of neuronal GSH can result in increase of neuronal NO synthase activity and cell death^[11]. In clinics, patients with peptic ulcer show a decreased gastric GSH^[13]. The cytoprotection of GSH on gastric mucosal injury induced by ethanol in rats has also been documented^[37].

Our previous report demonstrated that increase in oxyradical generation as indicated by augmentation of

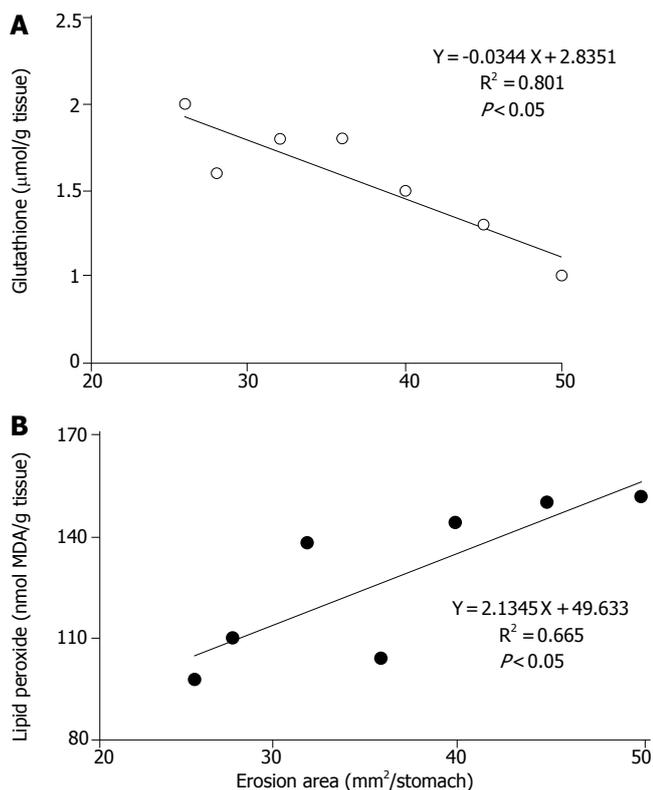


Figure 2 Relationship between gastric mucosal ulceration and mucosal GSH levels as well as between gastric mucosal ulceration and lipid peroxide generation in gastric juice-irrigated stomachs of rats with ischemic brain. Rat stomachs were irrigated for 3 h with simulated gastric juice.

concentration of mucosal LPO, the oxyradical metabolite, was parallel to the elevation of histamine concentration in endotoxemic rats^[14]. In the present *in vivo* study, increased mucosal histamine release may not only be derived from gastric mast cell *per se*, but also may be from those of other organs, including liver, lung, kidney or peritoneal tissues via circulation. Oxyradicals can directly attack mast cells, the predominant storage site for histamine, and cause cell degranulation. In turn, gastric mucosal histamine concentration is elevated. Since inflammation is greatly associated with oxyradical formation^[23], and increase in mucosal histamine concentrations may exacerbate tissue inflammation, it is likely that the aggravation of mucosal inflammation by increased histamine concentrations can produce more oxyradicals. The increased histamine found in rats with ischemic brain could be attenuated by allopurinol or exogenous GSH, implying that enzymology of oxyradical formation was involved in the metabolism of histamine. Taken together, increased histamine release and oxyradical generation in the gastric mucosa might result in hemorrhage and erosion in rats with ischemic brain. We have previously reported that activation of histamine H₁ and H₂ receptors is important in the formation of gastric hemorrhage and ulcer in septic rats^[15].

In the present study, L-arginine produced a dose-dependent increase in mucosal GSH levels and an attenuation of mucosal histamine and LPO concentrations in rats with ischemic brain. Gastric hemorrhage and stomach erosions were also remarkably ameliorated. Apart from its roles in protein biosynthesis and as an

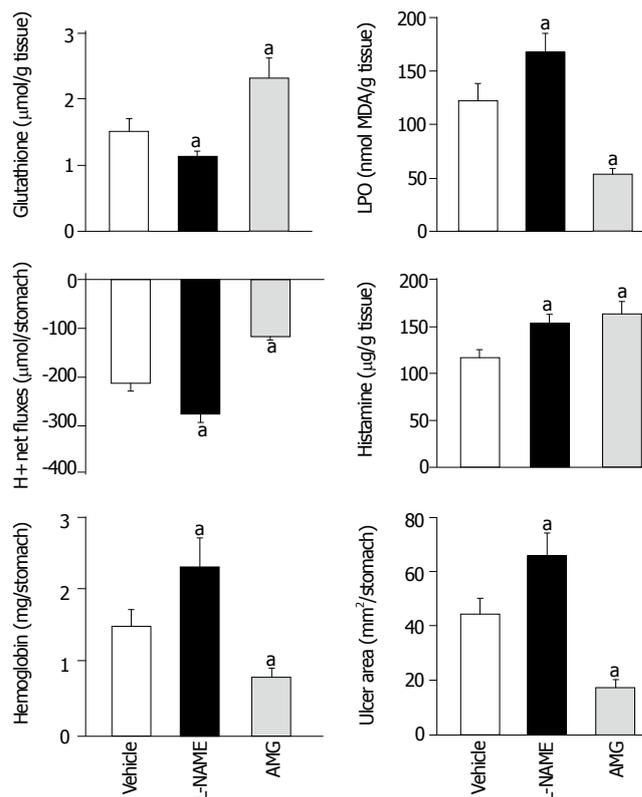


Figure 3 Effects of L-NAME and AMG on various gastric parameters in gastric juice-irrigated stomachs of rats with ischemic brain. Data are expressed as mean ± SE (n=8). ^aP < 0.05 vs vehicle.

intermediate in the urea cycle, L-arginine is a substrate for NO production and phosphocreatine synthesis as well as a precursor to proline, glutamate and putrescine via ornithine. NO is formed from L-arginine by NO synthases (NOS). Three isoforms of this enzyme have been identified; two are constitutively expressed and one is iNOS^[7]. NO has dual effects on living cells. One is defensive and the other is injuring. Over-expression of NO may cause cell damage and dysfunction that plays a pivotal role in many diseases, including intestinal ischemia-reperfusion^[20], hypertension^[30], inflammation^[6], cell death^[2]. On the other hand, physiological NO can protect cells against oxidative damage^[38]. It also mediates many of the manifestations of septic shock, including hemodynamic instability^[9]. L-arginine has been successfully used to increase vascular dilation and basal NO synthesis, and thereby reducing ischemia/reperfusion-induced injury^[31]. Oral supplementation of L-arginine in hypercholesterolemic patients with endothelial dysfunction increased endothelial-dependent dilation of the bronchial artery during increased cardiac output^[4]. L-arginine also promotes mucosal repair after intestinal ischemia/reperfusion^[35] and relieves mucosal damage during endothelin-induced ulceration^[21]. These protective effects of L-arginine are abrogated by L-NAME, a non-selective inhibitor of NO synthase. It has been reported that L-arginine can enhance mucosal growth and repair by supporting polyamine synthesis^[27]. Also, it can accelerate healing of gastric erosions in rats and this effect could be attributed to the stimulation of gastric mucosal blood

flow through increased NO synthesis and stimulation of mucosal growth^[3]. Altogether, these beneficial effects of L-arginine may account for the protective effects of L-arginine on gastric hemorrhagic erosions in rats with ischemic brain. In the present study, the attenuation of hemorrhage and stomach erosion in rats with ischemic brain by parental challenge of L-arginine may be associated with NO formation via arginine-NO-pathway.

The present study also showed that AMG significantly reduced gastric hemorrhagic ulcer in rats with ischemic brain. We also observed inhibition of lipid peroxide generation and augmented GSH level. However, it augmented mucosal histamine concentration. AMG is a relatively selective inhibitor of iNOS, but also is a specific inhibitor of histaminase, an enzyme that breaks down histamine. The elevated histamine by AMG might be a net result of its inhibition on histaminase and iNOS. In fact, specific inhibition of iNOS that is up-regulated in many tissues during diseases has been explored as a therapy for diseases. AMG has been shown to prevent the endotoxin-induced impairment of vascular reactivity when administered *in vivo*^[19]. It has also been reported to reduce streptozotocin-induced hyperglycemia^[34] and brain damage produced by occlusion of the middle cerebral artery in rats^[29]. Taken together, iNOS may play an important role in the formation of hemorrhagic erosion in rats with ischemic brain.

In the present study, L-NAME failed to protect gastric mucosal damage in rats with ischemic brain. On the contrary, it reversed mucosal protective effect of L-arginine. The result might be due to the inhibition of constitute NOS by L-NAME. In fact, NO synthesis may provide protection to tissues after ischemia/reperfusion-induced injury of the tissue^[22]. Document indicates that inhibition of NO synthesis by L-NAME increases ischemia/reperfusion-induced injury, which can be reversed by perfusion with NO or its donors that can decrease injury and neutrophil accumulation^[27].

In conclusion, gastric oxidative stress and excess iNOS production may play a pivotal role in modulation of hemorrhagic erosion through increased acid back-diffusion, histamine release and oxyradical generation as well as decreased GSH levels in rats with ischemic brain.

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CLINICAL RESEARCH

Distal small bowel motility and lipid absorption in patients following abdominal aortic aneurysm repair surgery

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Lipid absorption data was available for seven patients on d 1 and four patients on d 3 post surgery. In patients, absorption on d 1 post-surgery was half that of healthy control subjects (AUC $^{13}\text{CO}_2$ 1323 ± 244 vs 2646 ± 365 ; $P < 0.05$, respectively), and was reduced to the one-fifth that of healthy controls by d 3 (AUC $^{13}\text{CO}_2$ 470 ± 832 vs 2646 ± 365 ; $P < 0.05$, respectively).

CONCLUSION: Both proximal and distal small intestinal motor activity are transiently disrupted in critically ill patients immediately after major surgery, with abnormal motility patterns extending as far as the ileum. These motor disturbances may contribute to impaired absorption of enteral nutrition, especially when intraluminal processing is necessary for efficient digestion.

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Key words: Critical illness; Small intestine; Motility; Lipid absorption

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Abstract

AIM: To investigate distal small bowel motility and lipid absorption in patients following elective abdominal aortic aneurysm (AAA) repair surgery.

METHODS: Nine patients (aged 35-78 years; body mass index (BMI) range: 23-36 kg/m²) post-surgery for AAA repair, and seven healthy control subjects (20-50 years; BMI range: 21-29 kg/m²) were studied. Continuous distal small bowel manometry was performed for up to 72 h, during periods of fasting and enteral feeding (Nutrison®). Recordings were analyzed for the frequency, origin, length of migration, and direction of small intestinal burst activity. Lipid absorption was assessed on the first day and the third day post surgery in a subset of patients using the ^{13}C -triolein-breath test, and compared with healthy controls. Subjects received a 20-min intraduodenal infusion of 50 mL liquid feed mixed with 200 μL ^{13}C -triolein. End-expiratory breath samples were collected for 6 h and analyzed for $^{13}\text{CO}_2$ concentration.

RESULTS: The frequency of burst activity in the proximal and distal small intestine was higher in patients than in healthy subjects, under both fasting and fed conditions ($P < 0.005$). In patients there was a higher proportion of abnormally propagated bursts (71% abnormal), which began to normalize by d 3 (25% abnormal) post-surgery.

INTRODUCTION

Enteral feeding reduces both morbidity and mortality in critically ill patients^[1]. A number of factors, including high gastric aspirate volumes^[1,2], reduce the effectiveness of enteral feeding in critically ill patients by delaying its commencement or continuation. Slow gastric emptying is a major cause of difficulties with intragastric delivery of feeds, and occurs in 30-50% of mechanically ventilated patients^[3]. Prokinetic stimulation of gastric motility or bypassing the stomach with jejunal feeding tubes overcomes some of these difficulties^[4]. However, gastrointestinal symptoms such as diarrhea, abdominal distention, vomiting, and regurgitation remain common in enterally fed, critically ill patients^[2]. The motor mechanisms responsible for these symptoms are poorly understood, largely because data on small intestinal motility in critically

ill patients are very limited.

Previous studies have shown that the patterning of interdigestive motor activity in the proximal small intestine is frequently disturbed in the critically ill. Bursts of abnormally propagated pressure waves that resemble phase III of the migrating motor complex are present, and persist during enteral feeding¹⁵⁻⁷¹. The potential impact of this motor disturbance on nutrient absorption in the critically ill has not been assessed. We have previously shown in healthy human beings that lipid absorption is reduced when phase III activity is stimulated during enteral feeding¹⁸. Previous manometric studies in critically ill patients have been limited to the proximal and mid small intestine¹⁵⁻⁷¹. It is unknown whether the motor changes observed proximally are present further down the gut. If such abnormalities prevail along the full length of the intestine, they are likely to have a greater negative impact on the success of enteral feeding.

In this study, we have recorded intraluminal pressure patterns along the distal small intestine in a group of patients immediately after abdominal aortic aneurysm (AAA) repair surgery, and for the following 3 d. In a subset of patients, these findings were correlated with the measurements of lipid absorption using the ¹³C-triolein breath test¹⁹.

MATERIALS AND METHODS

Subjects

Nine patients (35-78 years: body mass index (BMI) range 23-36 kg/m²; APACHE II score¹⁰⁰ range 6-33) participated in the study following elective AAA repair surgery at Saint Andrew's Hospital, Adelaide during their post-surgical admission to the intensive care unit (ICU). All patients gave written informed consent before their surgery. Patients who had previously undergone upper gastrointestinal tract surgery or who were on medication known to affect gastrointestinal motility (other than the usual post-surgical drugs e.g. opiate analgesics) were excluded from the study.

Seven healthy control subjects (20-50 years: BMI range 21-29 kg/m²) were studied. Individuals with a history of cardiovascular, respiratory or gastrointestinal disease including severe anemia, eating disorders, diabetes mellitus or other serious past medical history were excluded. Subjects were not permitted to use medication (apart from paracetamol) in the two weeks before the study. Subjects gave written informed consent prior to entering the study.

The protocol was approved by the human research ethics committees of Saint Andrew's Hospital and the Repatriation General Hospital South Australia.

Manometric recordings

In all the subjects, small intestinal pressure waves were measured using a 200-cm-long, purpose-built silicone rubber multilumen assembly (outer diameter 4 mm) (Dentsleeve, Adelaide, South Australia), incorporating 16 pressure recording channels (inner diameter 0.4 mm). The two most proximal side-holes were spaced 5 cm apart and the following 14 at 12.5-cm intervals. A single larger channel of the assembly (inner diameter 0.9 mm, located 142 cm from the distal tip) was used to deliver enteral

Table 1 Time periods for manometry data analysis in patients following admission to the ICU

| Description | t(post surgery)/h | Feeding condition | Ventilation condition | n |
|----------------------|-------------------|--------------------|-----------------------|---|
| Early ventilation | 1-5 | No enteral feeding | Ventilation | 9 |
| Early post-operative | 20-24 | No enteral feeding | No ventilation | 7 |
| Early feeding | 29-33 | Enteral feeding | No ventilation | 8 |
| Late feeding | 53-57 | Enteral feeding | No ventilation | 8 |
| Late post-operative | 68-72 | No enteral feeding | No ventilation | 9 |

feeds into the distal duodenum. Correct positioning of the assembly was facilitated by small weights and a balloon (7 mL) located at the tip of the assembly.

All manometric lumina were perfused with degassed distilled water at a constant rate of 0.05 mL/min. This resulted in a total daily infusion volume of 1150 mL/d of water, which was accounted for in the patients' daily fluid, electrolyte, and nutrient requirements. Pressures were measured via external transducers (Abbott Critical Care Systems, North Chicago, IL, USA) and recorded onto a Power Macintosh G3 computer using MPS 100 software (Biopac System Inc., Santa Barbara, CA, USA).

Protocol

Patients were studied continuously over 3 d immediately following surgical repair of AAA. The manometric assembly was passed transnasally and its tip positioned in the stomach prior to surgery. The assembly incorporated a silicone rubber balloon at its distal tip that when inflated allowed the surgeon to palpate the tip of the catheter in the stomach. At the time of surgery, the assembly was passed through the pylorus, along the duodenum, jejunum, and ileum by manipulation of the inflated balloon by the surgeon, and by intrinsic motor activity. The final position of the assembly was determined by routine abdominal X-ray.

Measurement of intraluminal pressures commenced immediately after the arrival of patients in the ICU and continued for approximately 72 h. Patients were fasted for the first 24 h post surgery (d 1), during which low-pressure suction was used to aspirate gastric contents. On d 2, enteral feeding (Nutrison[®], Nutricia, Zoetermeer, Holland; 40-80 mL/h) was commenced via the infusion channel incorporated into the manometric assembly. The effects of enteral feeding on small intestinal motility were assessed in two time windows: (1) immediately after commencing enteral feeding (d 2; early feeding period, n=8) and (2) over the following 24 h (d 3; late feeding period, n=8). These data were averaged to obtain final feeding data (Table 1).

Healthy subjects arrived at the Repatriation General Hospital Gastrointestinal Ward after an overnight fast from 2000 h (except for water). The manometric assembly was introduced into the stomach *via* an anesthetized nostril (Lignocaine 50 g/L, PaedPharm, Australia). The subject was then positioned in the right lateral position until the tip of the assembly had passed beyond the pylorus. Progression of the assembly along the small intestine was assisted by inflation of the catheter balloon. Correct positioning of the assembly occurred when only the two most proximal side-holes remained in the gastric antrum,

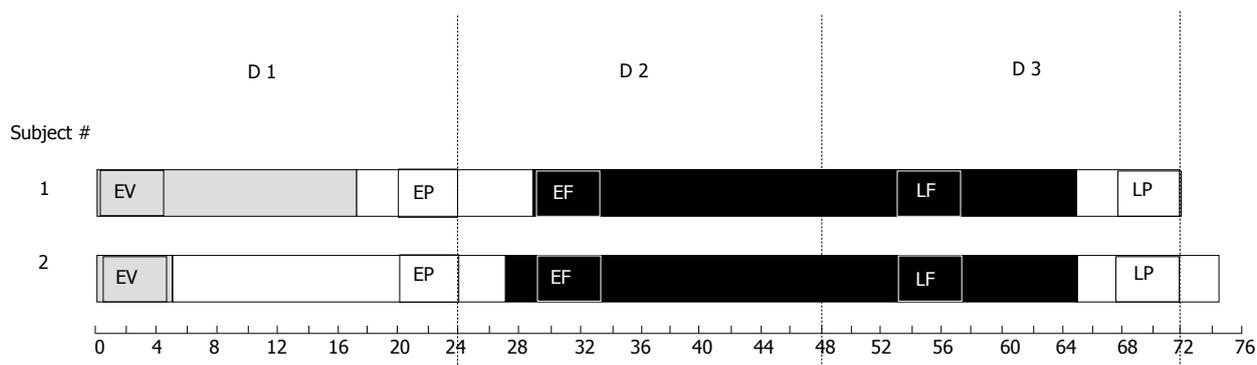


Figure 1 Schematic representation of manometry recording periods in two patients: ventilated and fasting (gray), non-ventilated and fasting (white), non-ventilated and feeding (black); and analysis periods: early ventilation (EV) (1-5 h), early post-operative (EP) (20-24 h), early feeding (EF) (29-33 h), late feeding (LF) (53-57 h) and late post-operative (LP) (68-72 h).

which was verified by the analysis of pressure wave frequencies^[13]. Side holes located within the first 25 cm of the small intestine were considered to be in the duodenum, side holes in the next 120 cm were defined to be in the jejunum, and the remaining side holes were considered to be in the ileum.

Manometric recordings commenced immediately following the correct placement of the assembly and continued for 72 h. Pressures were measured during fasting (0-24 h) and enteral feeding (Nutrison[®], Nutrica, Zoetermeer, Holland; 80 mL/h) (24-72 h).

Lipid absorption test

Lipid absorption was assessed using the ¹³C-triolein breath test^[9]. In patients, absorption was evaluated on d 1 ($n=7$) and d 3 ($n=4$) post surgery. Absorption data from both days were compared to healthy subjects ($n=7$). After a minimum of 4 h fasting, 50 mL of a mixed nutrient liquid (Nutrison[®], Nutrica, Zoetermeer, Holland) was mixed with 200 μ L of [1,1,1-¹³C] triolein (99%, 200 mg) (Cambridge Isotope Laboratories, Andover, MA, USA) and infused intraduodenally over 20 min. End-expiratory breath samples were collected at baseline (5 min prior to label administration) and every 30 min thereafter for 6 h^[9]. For patients, a purpose-built connector on the ventilator tube was used to collect the breath samples directly into 10 mL glass tubes (Exetainer[®], Buckinghamshire, UK). Healthy subjects were asked to exhale through a straw into the collection tubes. Breath samples were analyzed for ¹³CO₂ concentration using an isotope ratio mass spectrometer (Europa Scientific, ABCA model 20/20, Crewe, UK). The values obtained were converted to percent dose recovery per hour, and used to calculate the area under the CO₂ recovery curve (AUC ¹³CO₂).

Analysis of manometry

Manometric data obtained from patients were analyzed during five 4-h periods that corresponded to different stages within the 72 h post surgery: early ventilation (0-5 h), early post-operative (20-24 h), early feeding (29-33 h), late feeding (53-57 h), and late post-operative (68-72 h) (Table 1 and Figure 1). For healthy subjects, recordings were analyzed during fasting (d 1; 0-24 h) and enteral feeding (d 3; 48-72 h).

Small intestinal burst activity

Motility recordings were analyzed manually to determine the frequency, site of origin (proximal/not proximal), distance of propagation and direction of travel of small intestinal burst activity. A burst was defined as the presence of ≥ 10 pressure waves per minute for at least 2 min, which migrated sequentially over four or more side-holes^[11]. A threshold pressure wave amplitude >1.33 kPa measured from the end inspiratory pressure level to peak pressure was set to avoid confusion with respiratory pressure oscillations. Changes in pressure due to physical strains, which were recognized by having the same timing and amplitude in all channels, were excluded from the analysis. The spatial patterning of propagation of each burst sequence was classified as normal (antegrade, sequential) or abnormal (retrograde, bi-directional, simultaneous or multi-origin onset).

Statistical analyses

Analyses were performed using Statview Version 4.5 (Abacus Concepts Inc., CA, USA) software. The following parameters were analyzed: burst frequency (number of bursts per hour), percentage of normal bursts, percentage of bursts to start proximally, and the percentage of bursts to reach the ileum. Data were assessed using analysis of variance (AVOVA) to compare the data from different fasting periods. The Mann-Whitney *U* test was used to compare fasting and fed data, as well as patient and healthy data. Data for triolein absorption was compared using the Mann-Whitney *U* test. Data were expressed as mean \pm SE. A $P < 0.05$ was considered statistically significant.

RESULTS

The study protocol was well tolerated by all the subjects. Four patients reported minor local discomfort in the throat due to intubation of the manometry extrusion. The median APACHE II score^[10] in patients was 11 (range 6-33). Major concomitant diseases were coronary artery disease ($n=7$), hypertension ($n=2$), chronic pulmonary disease ($n=3$) and diabetes mellitus ($n=1$). Manometric recordings were performed continuously for 72 h after surgery (range 66-121 h). Patients received mid-thoracic

Table 2 Site of origin and distance of propagation of small intestinal burst activity during fasting and enteral feeding (mean±SE)

| Time period | % Bursts to start in the antrum | % Bursts propagated to the ileum |
|------------------------------------|---------------------------------|----------------------------------|
| Fasting | | |
| Patient: Early ventilation (1-5 h) | 70.0±5.6 ^b | 62.7±9.3 |
| Early post-operative (20-24 h) | 70.1±6.2 ^b | 35.2±8.6 |
| Late post-operative (68-72 h) | 75.5±8.2 ^b | 33.8±8 ^c |
| Healthy | 76.6±14.3 | 43.7±17.6 |
| Enteral feeding | | |
| Patient | 65.3±7.6 ^a | 53.9±12.4 |
| Healthy | 75.0±11 | 29.2±12.0 |

^a $P < 0.05$ vs healthy fed, ^b $P < 0.01$ vs healthy fast, ^c $P < 0.05$ vs patient early ventilation.

epidural anesthesia with 1.25 g/L marcain and 15 mg/L fentanyl for a median time of 66 h (range 21-75 h) after the operation.

Frequency of burst sequences

The frequency of small intestinal bursts was higher in patients under both fasting and feeding conditions, compared to healthy control subjects (Figure 2). During fasting, the frequency of bursts was six times greater in patients during the early ventilation period (0-5 h), compared to healthy controls ($P < 0.0001$, Figure 2). By day 3 (68-72 h), the burst frequency in patients had reduced, but was still three times greater than healthy subjects ($P < 0.0001$). Feeding suppressed the frequency of bursts by 23% in patients, compared to 50% in healthy subjects (Figure 2).

Site of origin

The percentage of bursts originating in the antrum was less in patients during fasting ($P < 0.01$) and enteral feeding ($P < 0.05$), compared to healthy control subjects (Table 2). Feeding had no effect on the number of bursts originating in the antrum in patients (70.0%±6.0% vs 65.0%±8.0%, early post-operative vs feeding) or healthy subjects (76.6%±14.3% vs 75.0%±11%, fasting vs feeding).

Distance of propagation

A greater proportion of bursts reached the ileum in patients under both fasting and fed conditions, compared to healthy subjects (Table 2). In patients, the percentage of bursts reaching the ileum was higher during early ventilation (1-5 h) compared to the late post-operative (68-72 h) period ($P < 0.05$, Table 2).

Patterning of burst migration

Bursts migrated normally in healthy subjects during both fasting and feeding conditions. In patients, the majority of bursts (71%) were abnormally propagated during the early ventilation period (1-5 h) (Figure 3); this proportion decreased over time so that by d 3 only 25% of bursts migrated abnormally ($P < 0.01$; early ventilation vs late post-operative) (Figures 3 and 4).

Lipid absorption

Absorption data were available for seven patients on d 1

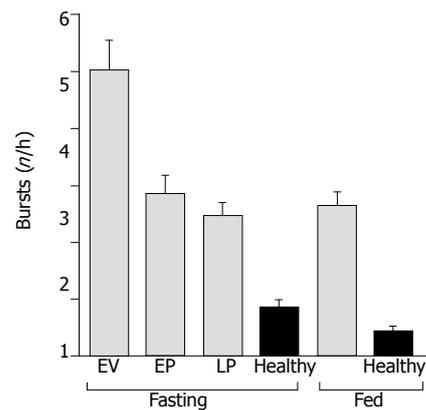


Figure 2 Bursts recorded during fasting (early ventilation (EV) (1-5 h), early post-operative (EP) (20-24 h) and late post-operative (LP) (68-72 h) and enteral feeding, in patients (gray shading) and healthy controls (black shading) ($n = 7$). Data are mean±SE. $P < 0.0001$ vs healthy fasting, $P < 0.0001$ vs healthy fed, early ventilation ($n = 9$), early post-operative ($n = 7$), enteral feeding ($n = 8$), late post-operative ($n = 9$).

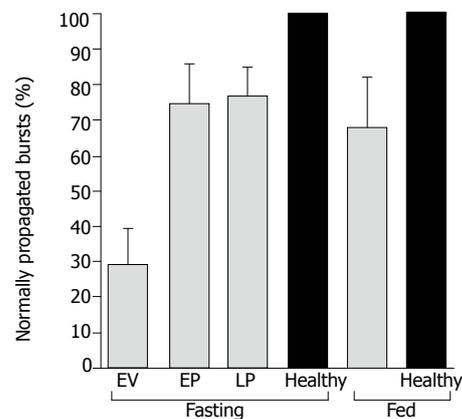


Figure 3 Normal burst activity (% of total burst activity) recorded during fasting (early ventilation (EV) (1-5 h), early post-operative (EP) (20-24 h) and late post-operative (LP) (68-72 h) and enteral feeding in patients (gray shading) and healthy control subjects (black shading) ($n = 7$). Data are mean±SE. $P < 0.01$ vs late post-operative, early ventilation ($n = 9$), early post-operative ($n = 7$), enteral feeding ($n = 8$), late post-operative ($n = 9$).

post surgery, and four patients on d 3 due to minor tube displacement. Recovery of $^{13}\text{CO}_2$ was slower in patients on d 1 post surgery than healthy control subjects (AUC $^{13}\text{CO}_2$ 1323±244 vs 2646±365; $P < 0.05$; respectively). In the four patients for whom data were available, $^{13}\text{CO}_2$ recovery on day 3 post-surgery was one-fifth of the healthy controls (470±832 vs 2646±365; $P < 0.05$; respectively). In patients, there was no difference in the recovery of $^{13}\text{CO}_2$ between d 1 and 3 post surgery (Figure 5).

DISCUSSION

This is the first study to examine distal small intestinal motor activity in critically ill post-operative patients. The results show a transient disruption in the patterning of small intestinal motility extending as far as the ileum, in the immediate post-operative period. Ileal motor activity was present early after surgery, but was characterized by frequent bursts of abnormally propagated pressure waves,

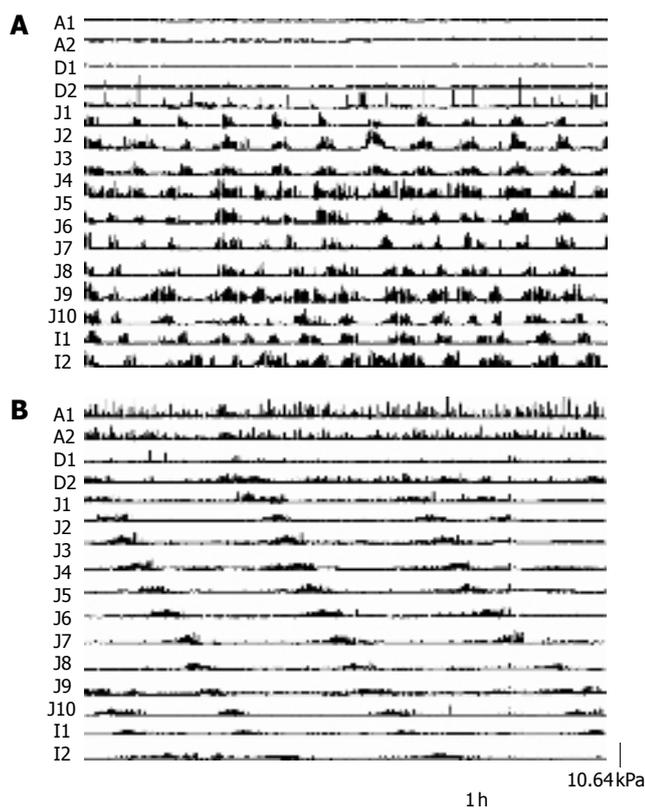


Figure 4 Sample patient small intestinal manometric tracing during fasting. **A:** d 1 showing characteristic disordered burst activity with simultaneous onset and retrograde propagation; **B:** d 3 bursting activity is less frequent and predominantly antegrade.

as seen in the proximal small intestine. These bursts of activity were not abolished by duodenal nutrient infusion. Previous studies in critically ill patients have demonstrated a disruption in small intestinal motor activity, with frequent and irregular bursts of activity in the duodenum and proximal jejunum^[5,6]. The distance that these motor events migrated along the intestine was previously unknown. The recording sites in earlier studies were limited by lack of access to the distal intestine. In addition, the number of pressure sensors was restricted by the volume of perfusate necessary to undertake the recordings. The novel silicone rubber catheter extrusion used in the present study allowed very low rates of manometric perfusion so that recordings from the distal small bowel could be made from 16 sites simultaneously, with a total delivery of perfusate per 24 h of approximately 1 L. This is within the allowable tolerance for fluids in critically ill patients and minimizes the risk of fluid overload. Our data thus extends previous findings by demonstrating that the motor disruption in critically ill patients is generalized at least as far as the proximal ileum. In health, phase III activity has a maximum incidence in the proximal jejunum, with only a minority of MMCs reaching the ileum^[12].

As previously reported, motor activity in critically ill patients is frequently abnormal^[5,6]. In the present study, bursts of phase 3-like activity were seen extending to the ileum within the first 24 h post-surgery. The spatial organization of these bursts, however, were markedly abnormal, with both simultaneous onset and progression in a ret-

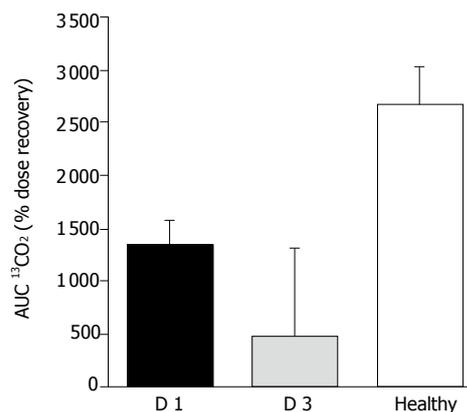


Figure 5 Area under the curve for percentage dose recovery of ¹³CO₂ until 6 h after the start of the intraduodenal infusion on d 1 ($n=7$) and d 3 ($n=4$) post surgery in AAA repair patients and in healthy subjects ($n=8$). $P<0.05$ vs healthy. Data are mean \pm SE.

rograde fashion, rather than a sequential migration down the gut that is typical of the fasting motor pattern. Over the subsequent 48 h, the organization of the bursts began to normalize and their frequency was reduced. However, the occurrence of bursts did not diminish during nutrient infusion, in contrast to the motor activity recorded from healthy subjects.

Enteral feeding remains as the preferred route of nutrition for critically ill patients^[1]; however, effective delivery of feeds is often hampered by delayed gastric emptying^[3,5]. Gastric dysmotility and stasis are likely to impair emptying both directly by the prevention of nutrient transit, and indirectly by the stimulation of feedback mechanisms that retard gastric motility. Some of these problems may be overcome by the use of post-pyloric feeding tubes; however, symptoms such as bloating, distension, and diarrhea remain common in patients receiving enteral nutrition. The effect of disordered small intestinal motility on the well-being of the patient is unclear.

There are limited data on the efficiency of the absorptive process in the critically ill. Singh *et al*^[13] have previously demonstrated a severe depression in gut absorptive capacity for D-xylose in patients suffering from sepsis or trauma^[13], particularly during the first 3-4 d of admission. Although the number of patients is limited in the present study, the results provide some insights into lipid absorption during critical illness. Our data suggest that lipid absorption may be impaired for at least 3 d following major surgery. The mechanisms responsible for these findings are unclear. It is likely that mucosal ischemia contributes to the disruption of the absorption process. However, the demonstration that small intestinal dysmotility extends to the ileum may also have consequences for absorption in these patients. The failure of conversion from a fasting to a fed motor pattern may disrupt the mixing of substrate with digestive enzymes that is normally seen with postprandial motility. We have previously shown a reduction in lipid but not glucose absorption when erythromycin is used to stimulate small intestinal burst activity in healthy human beings^[8]. The motor patterns recorded at 72 h in the present study are likely to increase the rate of nutrient transit through the gut. Accelerated transit has been associated

with reduced absorption in patients with ileostomies^[14]. Changes in small intestinal motor function may therefore have important implications for the absorption of enteral nutrition in these patients. Further studies are necessary to examine the relative contributions of mucosal and motor dysfunction in the critically ill.

The mechanisms underlying the motor patterns recorded in the present study are unknown. Although all patients had manipulation of the bowel during the placement of the catheter, this is unlikely to explain the findings, as similar motor patterns have been reported in the proximal intestine of critically ill patients who have not undergone surgery^[5,6]. It is also possible that drugs administered during routine clinical care may contribute to the observed motor dysfunctions. Opiates such as morphine are well recognized to produce burst activity. However, the majority of patients in our study received analgesia via an epidural catheter and required minimal or no systemic opiates during their ICU admission. Patients who required additional sedation for tolerance of mechanical ventilation usually received propofol. The effects of epidural anesthesia on motility are unclear. Although more rapid small intestinal transit has been reported when patients received epidural anesthesia instead of systemic opiates^[15], spinal or epidural anesthesia had no effect on orocecal transit times in healthy volunteers^[16]. It has been proposed that the fasting small intestine is under tonic inhibition by predominantly nitrergic mechanisms. It is thus possible that acute metabolic insults associated with critical illness disrupts these inhibitory pathways resulting in the release of motor activity, until control mechanisms recover.

In conclusion, we have shown that abnormal small intestinal motor activity in critically ill patients extends as far as the ileum. The disruption to the organization of fasting motor patterns has improved by d 3 post surgery, but the motor response to intestinal nutrients, and in particular, the conversion to postprandial motor activity remains impaired. The effects of these motor abnormalities on nutrient transit and absorption, together with their contribution to symptoms requires further study, as do the mechanisms underlying these dysfunctions.

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CLINICAL RESEARCH

Nitric oxide synthase and heme oxygenase expressions in human liver cirrhosis

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Abstract

AIM: Portal hypertension is a common complication of liver cirrhosis. Intrahepatic pressure can be elevated in several ways. Abnormal architecture affecting the vasculature, an increase in vasoconstrictors and increased circulation from the splanchnic viscera into the portal system may all contribute. It follows that endogenous vasodilators may be able to alleviate the hypertension. We therefore aimed to investigate the levels of endogenous vasodilators, nitric oxide (NO) and carbon monoxide (CO) through the expression of nitric oxide synthase (NOS) and heme oxygenase (HO).

METHOD: Cirrhotic ($n = 20$) and non-cirrhotic ($n = 20$) livers were obtained from patients who had undergone surgery. The mRNA and protein expressions of the various isoforms of NOS and HO were examined using competitive PCR, Western Blot and immunohistochemistry.

RESULTS: There was no significant change in either inducible NOS (iNOS) or neuronal NOS (nNOS) expressions while endothelial NOS (eNOS) was up-regulated in cirrhotic livers. Concomitantly, caveolin-1, an established down-regulator of eNOS, was up-regulated. Inducible HO-1 and constitutive HO-2 were found to show increased expression in cirrhotic livers albeit in different localizations.

CONCLUSION: The differences of NOS expression might be due to their differing roles in maintaining liver homeostasis and/or involvement in the pathology of cirrhosis. Sheer stress within the hypertensive liver may induce increased expression of eNOS. In turn, caveolin-1

is also increased. Whether this serves as a defense mechanism against further cirrhosis or is a consequence of cirrhosis, is yet unknown. The elevated expression of HO-1 and HO-2 suggest that CO may compensate in its role as a vasodilator albeit weakly. It is possible that CO and NO have parallel or coordinated functions within the liver and may work antagonistically in the pathophysiology of portal hypertension.

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Key Words: Liver cirrhosis; Nitric oxide synthase; Heme oxygenase; Gene expression; Competitive PCR

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INTRODUCTION

Liver cirrhosis is invariably associated with the incidence of hemodynamic disturbances manifested as portal hypertension and concomitant splanchnic vasodilation. Portal hypertension can be seen as a function of Ohm's law^[1]: $\Delta Pa = Q \times R$, where ΔPa is the intrahepatic pressure, Q is the blood flow from systemic circulation and R is the intrahepatic vascular resistance. Increasing either or both Q and R results in an elevation of portal pressure.

Increased hepatic vascular resistance is thought to be a consequence of architectural abnormalities comprising of vascularized connective tissue which interrupt the normal organization of liver parenchyma. This establishes vascular shunts between afferent (portal and hepatic artery) and efferent (hepatic vein) vessels^[2] leading to shunting. This condition is aggravated by the accommodation of the portal system to whatever is provided by the vasodilated splanchnic viscera.

Endogenous vasoconstrictors, such as endothelin and angiotensin II, mobilized by the onset or progression of cirrhosis may contribute to the increased hepatic vascular tone by the activation of hepatic stellate cells to a more contractile state^[1]. It follows that mitigation of the vascular dysfunction may be promoted by endogenous vasodilators, including nitric oxide (NO) and carbon monoxide (CO).

NO has an amazing repertoire of physiological functions for a molecule with a half-life <5 s, including

homeostasis and immunological defense mechanisms, within the liver. NO is synthesized from L-arginine by three known isoforms of nitric oxide synthase (NOS), namely neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS)^[1-4]. Under normal physiological conditions, only constitutively expressed eNOS is believed to be active in the liver and its low level of NO production suffices for regulating hepatic perfusion, preventing platelet adhesion, thrombosis and polymononuclear cell accumulation. In pathological conditions, such as sepsis, hepatitis and endotoxaemia, iNOS is activated and induced to produce sustained amounts of NO^[3].

Zakhary *et al*^[5] has suggested that CO and NO share contiguous, if not overlapping, areas of influence on vascular tone. Analogous to NOS, haem oxygenase (HO) exists in 3 isoforms: inducible HO (HO-1) and the 2 constitutively expressed HO (HO-2 and HO-3). CO, arising from the metabolism of haem by HO, has been found to attenuate sinusoidal tone and is responsible for the perfusion maintenance in normal liver^[5].

Current understanding of the contributions to the onset and/or progression of cirrhosis by NOS is not clear. Accordingly, this study aims to investigate the levels of NO and CO indirectly through the expression and distribution of NOS and HO in cirrhotic and non-cirrhotic human livers.

MATERIALS AND METHODS

Patients

Liver specimens were collected from 20 patients, including 10 males and 10 females (mean age 32.5 ± 25.5 years), who received orthotopic liver transplantation for end-stage liver diseases. Postoperative pathology reports confirmed the presence of cirrhosis. Samples with cirrhosis due to biliary obstruction or hepatic venous obstruction and not viral or alcoholic origin were studied. Twenty control liver tissues from 14 male and 6 female patients (mean age 57.2 ± 11.9 years) were obtained from partial hepatectomy surgery for malignant or other liver diseases (hepatocellular carcinoma, adenocarcinoma, leiomyosarcoma, gall bladder cancer infiltrating the liver, colonic carcinoma, Klaskin tumor). These controls were obtained from the outermost region of the tumor-free periphery from the excised lobe. The samples in our control group were ascertained to be cirrhosis-free.

Each patient provided a written informed consent before recruitment into the study. The protocol conformed to the ethical guidelines of the 1987 Declaration of Helsinki and those of the NMEC (National Medical Ethics Committee) - MOH (Ministry of Health) Ethical Guidelines on Research involving Human Subjects (1997).

All liver tissue samples were obtained intra-operatively and were snap frozen in liquid nitrogen not more than 20 min after excision and stored at -80°C until processed. Venous blood of patients was collected in EDTA-tubes and centrifuged at 3000 g for 10 min at 4°C . Plasma samples were aliquoted and stored at -20°C until use.

NO metabolites: Nitrite and nitrate (NOx -)

Plasma was analyzed for total NO metabolites using Total

NO kit (R & D Systems, Germany). Briefly, plasma was deproteinized by ultra-filtration. Enzymatic conversion of nitrate to nitrite by nitrate reductase preceded a colorimetric detection of nitrite as an azo dye product of the Griess reaction. Absorbance was read at 570 nm.

RT-PCR

Total RNA was extracted using TriZol Reagent (Invitrogen, Grand Island NY), according to the manufacturer's protocol. Reverse transcription (RT) was performed with oligo(dT) using Superscript II RNase H-reverse transcriptase (Invitrogen, Maryland). First strand cDNA synthesis was performed at 42°C for 1 h. RNA-cDNA hybrids were denatured at 72°C for 15 min. RT-products were stored at -20°C until use. PCR was performed using a DNA amplification kit (Qiagen, Germany) with Hybaid PCR express thermal cycler (UK).

Primer sequences were as follows: β -actin, 5' -GTGGGGCGCCCCAGGCACCA-3' (sense) and 5'-CTCCTTAATGTCACGCACGATTC-3' (antisense); iNOS, 5' -GTGGTCCAACCTGCAGGTCT-3' (sense) and 5'-CATAGCGGATGAGCTGAGCATT-3' (antisense); eNOS, 5' -GACATTGAGAGCAAAGGGCTGC-3' (sense) and 5' -CGGCTTGTCACTCCTGG-3' (antisense); nNOS, 5' -GCAACAGCGGCAATTTG-3' (sense) and 5'-GGTTGTCATCCCTCATCCGGCTG-3' (antisense); HO-1, 5' -CAGGCAGAGAATGCTGAGTTC-3' (sense) and 5' -GCTTCACATAGCGCTGCA-3' (antisense); HO-2, 5'-GCAATGTCAGCGGAAGTGGAA-3' (sense) and 5'-AAGTCACCTGAGGTGGTAGTT-3' (antisense); caveolin-1, 5'-CATCCCGATGGCACTCATCTG-3' (sense) and 5' -CACGGCTGATGCACTGAATCT-3' (antisense).

Predicted sizes for PCR products were 560 bp for β -actin, 187 bp for iNOS, 510 bp for eNOS, 387 bp for nNOS, 270 bp for HO-1, 1135 bp for HO-2 and 116 bp for caveolin-1. Each 25 μL of PCR reaction contained 0.4 $\mu\text{mol/L}$ of sense- and antisense primers, 2.5 $\mu\text{mol/L}$ of each dNTP, 0.5 units of Taq polymerase and 2.5 μL of cDNA, except for iNOS in which 5 μL was used. Annealing temperatures for β -actin, iNOS, eNOS, nNOS, HO-1, HO-2 and caveolin-1 were 55°C (25 cycles), 62°C (40 cycles), 60°C (30 cycles), 60°C (40 cycles), 62°C (30 cycles), 65°C (25 cycles) and 62°C (40 cycles), respectively. β -actin was used as a housekeeping gene.

Competitive PCR

Relative levels of mRNA expression can be semi-quantified by co-amplifying the cDNA with a known concentration of an internal competitor DNA. The competitor DNA, Mimic, is a non-homologous DNA fragment derived from the v-erb b gene to which a pair of gene-specific primers template had been added. The Mimic was constructed according to manufacturer's protocol (Clontech Laboratories, USA). A constant amount of cDNA was first amplified with a series of 10-fold dilution of its Mimic. This titration provided a fine-tuned 2-fold dilution series of Mimic. The amount of Mimic that obtained an optical density of 1:1 was then chosen to co-amplify with all the samples. After PCR, the products were resolved on a 18 g/L agarose gel and visualized by ethidium bromide. The ratio of optical density of target

against Mimic was then determined via the KODAK 1D Image Analysis Software (United States).

Western immunoblot

The expressions of NOS and HO isoforms in liver samples were measured by densitometry quantitation of immunoblots. Liver tissues were homogenized in a lysis buffer (0.5 g/L SDS in 0.1 mol/L Tris-HCl, pH 7.4) containing Complete Mini-tab EDTA proteinase inhibitor (Roche Biochemicals) and centrifuged at 13 000 g for 15 min at 4 °C. Then 100 µg of total protein was resolved on 80 g/L SDS-PAGE and transferred onto a PVDF-membrane using Mini Trans-Blot cell (BioRad Laboratories, California). Membranes were then blocked in 5% skim milk in phosphate buffered saline for 1 h at room temperature. After an overnight incubation at 4 °C using diluted monoclonal antibodies from Transduction Lab, (200x anti-iNOS, 1000x anti-eNOS, 2000x anti-nNOS, 1000x anti-HO-1 and anti-HO-2 and 500x anti-caveolin-1), the membranes were incubated with 1000x dilution of horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech). The blots were then visualized using ECL Plus Kit (Amersham) according to the manufacturer's instructions. Relative densities of the bands were analyzed using Kodak 1D Imaging System.

Immunohistochemistry (IHC)

Formalin-fixed and paraffin-embedded 4-µm thick sections of liver specimens were de-paraffinized in xylene (Merck, Germany) and rehydrated in decreasing concentration of ethanol. Antigen retrieval was carried out by heating the sections in 0.01 mol/L sodium citrate (pH 6.0). The sections were allowed to cool to room temperature before proceeding. Peroxidase Block (Amersham Pharmacia Biotech) was applied to inhibit endogenous peroxidase activity. Primary antibodies (same as those used in Western Immunoblot) were diluted (iNOS, eNOS, nNOS: 600x; HO-1, HO-2: 200x; caveolin-1: 400x) and incubated with the samples for 45 min at room temperature, followed by incubation with secondary antibody for 1 h at room temperature. Sections were then visualized with 3,3'-diaminobenzidine (DAB) and counter-stained with Harris' hematoxylin. The slides were dehydrated, cleared and mounted for viewing.

Statistical analysis

Graphpad Prism 4.0 was used in all statistical analysis. Student's *t* test for unpaired samples was used to compare data between cirrhotic and non-cirrhotic groups. Differences were considered statistically significant at values of $P < 0.05$.

RESULTS

Histopathology revealed micro- or macro-nodular cirrhotic morphology. Biliary cirrhosis secondary to biliary atresia or extrahepatic biliary obstruction resulting in cholestasis were the main causes of cirrhosis in our study group. The control group did not present cirrhotic morphology. All samples studied were found to be negative for hepatitis B antigen.

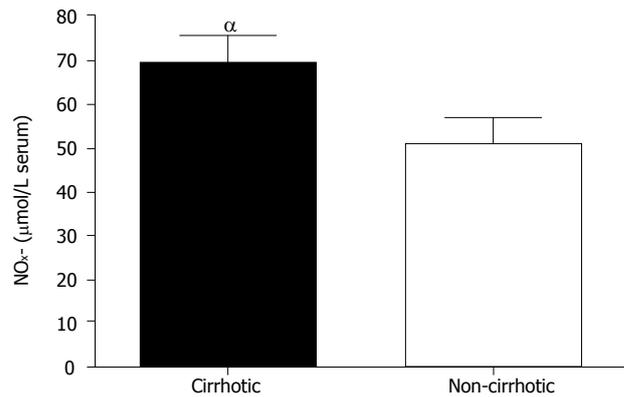


Figure 1 Total NO metabolites in plasma were measured from 20 cirrhotic patients and 20 non-cirrhotic controls. Cirrhotic patients NO_x⁻ levels were markedly higher at 69.72 ± 6.31 µmol/L serum; while non-cirrhotic patients NO_x⁻ levels were lower at 50.83 ± 5.67 µmol/L serum. * $P < 0.05$.

NO metabolites: Nitrite and nitrate (NO_x⁻)

Comparison of total NO_x⁻ in systemic plasma is shown in Figure 1. The data showed that cirrhotic patients had significantly higher NO_x⁻ levels (69.72 ± 6.31 µmol/L) as compared to the controls (50.83 ± 5.67 µmol/L) ($P < 0.05$).

Competitive RT-PCR

The mRNA levels of the various isoforms of NOS and HO were differentially expressed in cirrhotic and control groups (Figure 2). mRNA levels of nNOS and iNOS were not significantly different between the study and control groups (Figures 2A and 2B). Conversely, eNOS (Figure 2, $P < 0.05$) and both isoforms of HO (Figure 2D, HO-1: $P < 0.01$; Figure 2E, HO-2: $P < 0.01$) were significantly elevated in the cirrhotic group. Caveolin-1 mRNA expression was also significantly elevated ($P < 0.05$) in cirrhotic tissue (Figure 2F).

Western immunoblot

Protein levels, as semi-quantitated via densitometry of Western blot, agreed with our mRNA expression data. As shown in Figure 3A, nNOS was similar for both. Although iNOS seemed down-regulated in cirrhotic livers, it was not statistically significant (Figure 3B). We found significantly increased levels of eNOS (Figure 3C, $P < 0.05$), HO-1 (Figure 3D, $P < 0.05$) and HO-2 (Figure 3E, $P < 0.001$) in cirrhotic groups as compared to the control group. Likewise, caveolin-1 protein level was markedly increased ($P < 0.05$) in cirrhotic livers (Figure 3F).

IHC

Figure 4 shows liver sections of the cirrhotic study group (left panel) and the control group (right panel). nNOS (Figures 4A and 4B) was seen to have little or no variance in intensity and localization as compared to liver sections of the cirrhotic (Figure 4A) and non-cirrhotic (Figure 4B) groups. Generally, a uniform cytoplasmic stain was found in hepatocytes, while the Kupffer and sinusoidal cells remained unstained.

Similarly, iNOS (Figures 4C and 4D) was found in cytoplasmic regions of the hepatocytes. Kupffer cells, resident macrophages of the liver, were surprisingly not

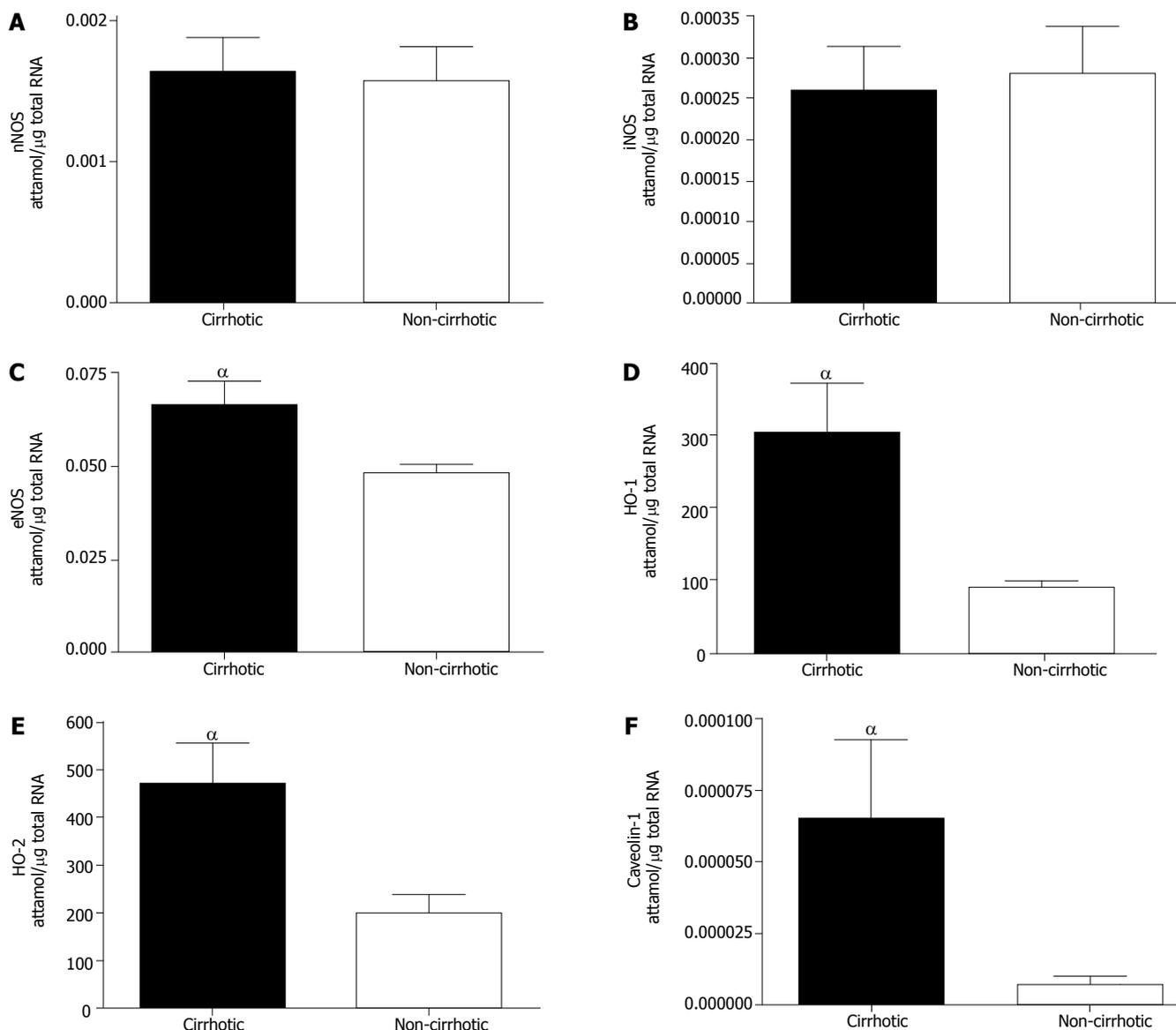


Figure 2 Differential mRNA expression in cirrhotic and non-cirrhotic patient livers were measured via competitive PCR. **A, B:** nNOS and iNOS showed no significant change; **C, D, E, F:** eNOS, HO-1, HO-2 and Caveolin-1 respectively showed significant increase when compared to non-cirrhotic mRNA. $^{\alpha}P < 0.05$.

stained. Overall, the cirrhotic group (Figure 4C) was ascertained to be comparable to the non-cirrhotic (Figure 4D) samples.

Conversely, eNOS (Figures 4E and 4F) displayed a distinct difference in localization in cirrhotic tissues. In the non-cirrhotic group (Figure 4F), eNOS was localized typically in endothelial cells as well as the cytoplasm of hepatocytes. Interestingly, translocation of eNOS from the cytoplasm to nuclei was evident in cirrhotic liver sections (Figure 4E). Also, noteworthy was the apparent attenuation of eNOS expression in endothelial cells, and at the same time enhanced staining was noted in hepatocytes in cirrhotic sections. Kupffer cells exhibited strong immunoreactivity to HO-1 in cirrhotic liver sections (Figure 4G), while control groups (Figure 4H) showed less intense immunoreactivity. HO-1 staining in Kupffer cells was noted to have a higher intensity in cirrhotic liver specimens compared to non-cirrhotic specimens. In contrast, HO-2 (Figures 4I and 4J) was found in the cytoplasm

of hepatocytes. Akin to HO-1, HO-2 was expressed at a higher level in cirrhotic liver as compared to the controls.

Moreover, we observed that caveolin-1 was more vividly stained in the study group (Figure 4K). They shared the same localization as that in the non-cirrhotic group (Figure 4L), namely the endothelial cells.

DISCUSSION

Several studies on diverse cirrhotic animal models have not reached agreement as to which isoform of NOS is the progressive/causative agent in portal hypertension^[6-8]. Studies on rat models have found a marked increase in iNOS mRNA and protein expression^[6-8], but upon treatment with specific eNOS inhibitors, injury exacerbation can be observed in spite of increased levels of iNOS^[9]. Ineffective selective inhibition of iNOS in ameliorating portal pressure underlined the importance of eNOS in intrahepatic haemodynamics^[6-9].

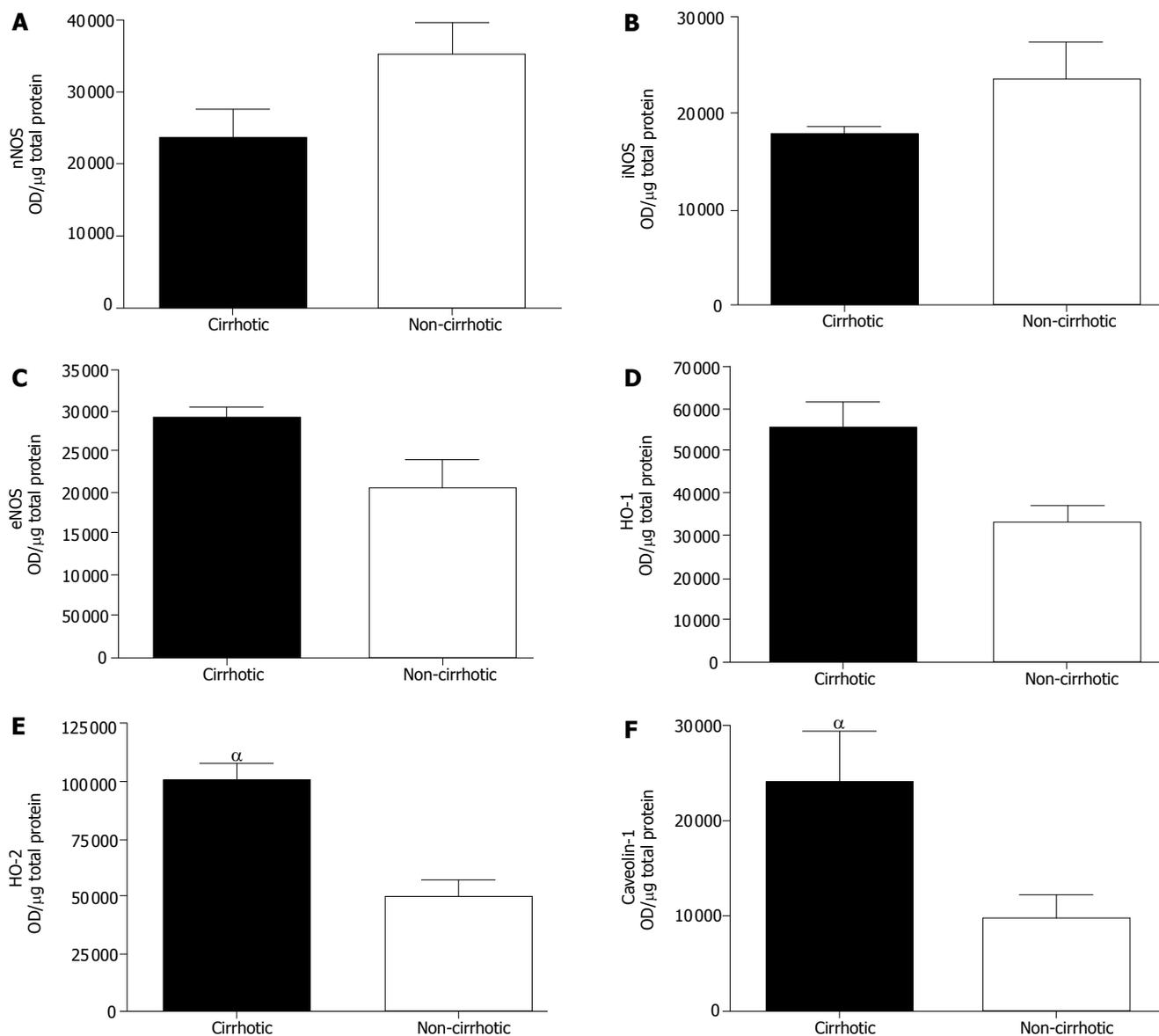


Figure 3 Protein expression in cirrhotic and non-cirrhotic patient livers were semi-quantitated via Western Blot. Results were tabulated and presented. Both nNOS (A) and iNOS (B) were unchanged. eNOS (C), HO-1(D), HO-2(E) and Caveolin-1(F) protein expression were significantly increased in cirrhotic tissue. OD represents optical density. $^{\alpha}P < 0.05$.

Similar to our results, McNaughton *et al*^[10] found no change in iNOS expression. Mohammed *et al*^[11] found that both eNOS and iNOS levels were elevated in cirrhosis, and systemic plasma NO_x^- increase in cirrhosis was mainly attributed to iNOS induction. Our data showed a similar increase in venous plasma NO_x^- . We hesitate to attribute this to increased NOS in the liver, although it remains a plausible source of NO.

In our patient group, eNOS and caveolin-1 were found to have greater expression, while no substantial changes in iNOS or nNOS were found. This induction of eNOS can be attributed to the shear stresses resulting from hypertension^[1,3]. Previously, we demonstrated that eNOS mRNA and protein levels in bile duct-ligated (BDL) rats were increased, yet activity declined by more than half^[12]. In support of this, Sarela *et al*^[13] showed that constitutive NOS activity was substantially lower in cirrhotic liver. In addition to the Ca^{2+} /calmodulin-dependent regulation,

caveolin-1 has been found to bind and inactivate NO production by eNOS^[14,15]. Caveolin-1 is known to be stimulated by cholesterol receptors^[14,15]. It is possible that the abnormal structure of the cirrhotic liver causes an induction of caveolin-1 or that the increasing fatty depositions common in cirrhosis contributes to this.

At rest, eNOS is bound to caveolin-1 and inactive. Upon stimulation, for example by shear stress, eNOS uncouples from caveolin-1 and translocates to the plasma membrane where it releases $\text{NO}^{[14]}$. Shear stress within the sinusoids of the cirrhotic liver presents unremitting stimuli for eNOS activation; which in turn is inhibited by increased caveolin-1 expression, reducing NO production intrahepatically. Our findings corroborate those of Yokomori *et al*^[15] that eNOS and caveolin-1 expressions increase in cirrhotic patients.

Not exclusive to endothelial cells, eNOS has been found in a variety of cell types, including mesangial cells,

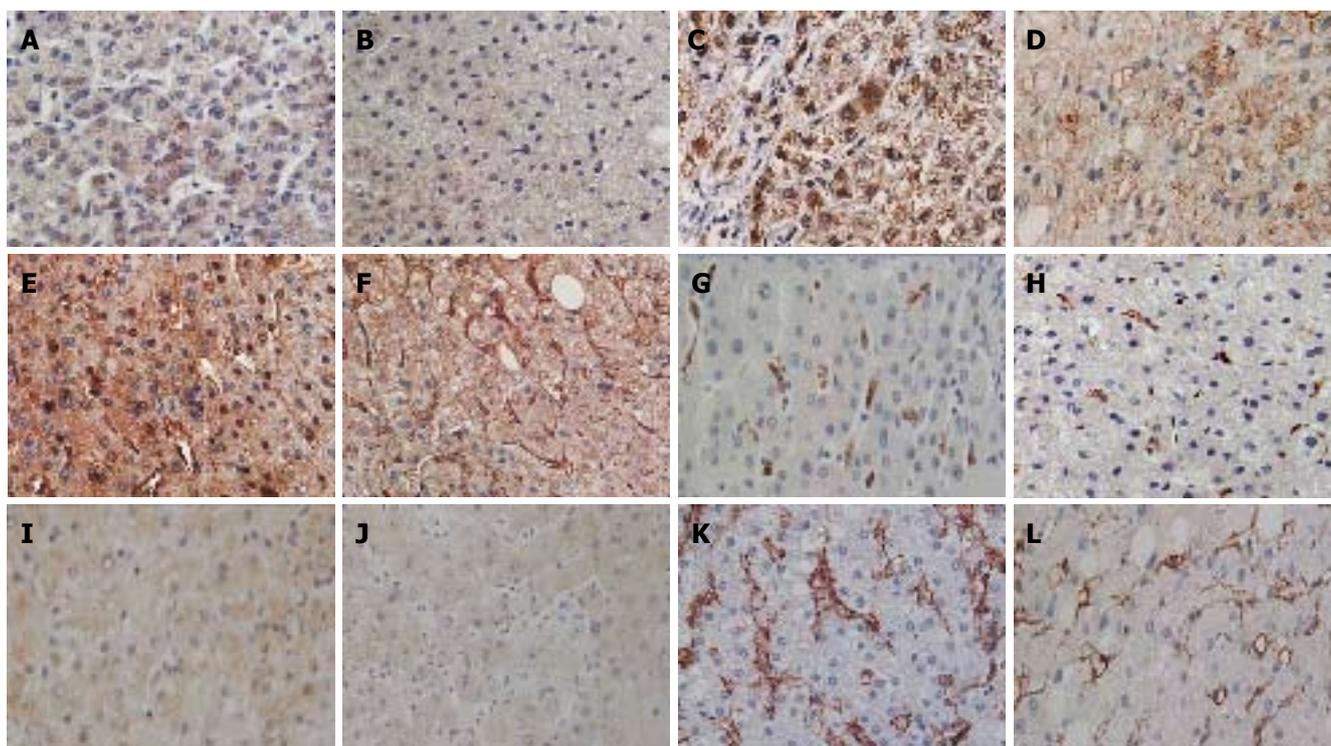


Figure 4 Immunohistochemistry of cirrhotic livers sections (left panel) and non-cirrhotic sections(right panel). **A** and **B**, show no difference in nNOS localization; likewise with iNOS as shown in **C** and **D**. eNOS was found to have translocated mainly to the nuclei in cirrhotic sections as evidenced in **E**, but found in hepatocyte cytoplasm and endothelial cells in non-cirrhotic as in **F**. In **G** and **H**, HO-1 is clearly found in macrophages with marked increase in cirrhotic sections (**G**). HO-2 was more highly expressed in the cytoplasm of hepatocytes in cirrhotic (**I**) than non-cirrhotic (**J**) tissues. Cirrhotic liver in **K** shows higher immunoreactivity to caveolin-1 that that of non-cirrhotic liver in **L**. In all IHC, monoclonal antibodies were used.

neurons^[8] and hepatocytes^[10]. We obviously showed that hepatocellular cytoplasmic localization of eNOS was not unique to cirrhotic livers, albeit the endothelial cells stained more intensely in non-cirrhotic livers (Figures 4E and 4F). This was also pointed out by McNaughton *et al.*^[10] where a similar translocation of eNOS to the nuclear periphery in cirrhotic sections was highlighted. The apparent attenuation of eNOS in cirrhotic endothelial cells may be due to the increased caveolin-1 expression in the same site. In normal liver, HO is accountable for the perfusion maintenance and sinusoidal tone^[5]. Our study showed that HO-1 and HO-2 had increased expression in cirrhotic livers. HO-1 seems to be exclusively expressed in Kupffer cells while HO-2 can be visualized in the hepatocyte cytoplasm. This was also evident in BDL rat, where HO-1 showed increased mRNA and protein levels^[16]. This corroborates results from Makino *et al.*^[17] as they found that HO-1 activity was markedly elevated in cirrhotic livers and inferred that the cirrhotic liver up-regulated HO activity mainly through the inducible isoform, HO-1. Induction of HO has also been attributed to shear stress^[17] and in the context of portal hypertension, up-regulation of HO-1 in Kupffer cells may serve as a local sensor of hemodynamics. In a study conducted by Sacerdoti *et al.*^[18] using CCl₄-induced cirrhosis in rats, HO-2 but not HO-1 was responsible for the splanchnic vascular hyporeactivity to vasoconstrictors. In our study, HO-2 was moderately increased in cirrhotic livers. However, the role of HO-2 in the pathophysiology of cirrhosis has not been well understood as yet. Our data suggest that it may have a

complementary function to that of HO-1 within the cirrhotic liver. Yet its obviously different locale from HO-1 implies that while it may have overlapping roles as HO-1, it may have distinctive functions that are as yet unknown or unassociated with cirrhosis.

NO has been found to stabilize HO-1 mRNA as well induce HO-1 in a cGMP-independent fashion^[19]. The increase of HO may be in part due to NO from splanchnic circulation or from the shear stress faced within the liver. These serve to provide CO, a vasodilator, in the absence of adequate amounts of NO from eNOS or iNOS. Increased production of vasodilator CO in Kupffer cells possibly aids the alleviation of shear stress. CO, unlike NO, is not a radical and is known to be a thousand-fold less potent than NO with respect to vasodilation and activation of cGMP^[16]. CO signaling may be particularly pertinent in conditions of oxidative stress^[17-20]. Exhaled CO has been found to be significantly increased in cirrhotic patients in comparison to healthy individuals^[20]. At low NO levels, such as in cirrhotic livers, CO can act a vasodilator through stimulation of soluble guanylate cyclase^[20]. Taken together, HO and its product CO may act to counteract portal hypertension in cirrhosis.

In summary, we demonstrated major differences between the expression and localization of eNOS and iNOS. Possibly these differences allude to their differing roles in maintaining liver homeostasis or involvement in the pathology of cirrhosis. The lack of difference in iNOS expression between cirrhotic and non-cirrhotic tissue suggests iNOS involvement in the pathology of other liver

diseases as truly normal liver tissues were not available. The increased expression of caveolin-1 within the cirrhotic liver is also suggestive of some form of regulation imposed upon eNOS. Whether this serves as a defense mechanism against further cirrhosis or as a result of cirrhosis with escalating portal hypertension as a consequence, is yet unknown. HO-1 and HO-2 are differentially expressed. It is possible that CO and NO have parallel or coordinated functions within the liver and may work together in the pathophysiology of portal hypertension.

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Expression and prognostic role of molecular markers in 99 KIT-positive gastric stromal tumors in Taiwanese

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immunoreactivity ($P=0.026$), high PCNA LI ($P=0.015$) and male gender ($P=0.036$) were six predictors for early disease recurrence. Subsequent multivariate analysis revealed that mitotic counts, tumor size, and p53 immunoreactivity were three independent prognostic factors for both disease free and overall survival of patients. By combination of three independent prognostic factors for grouping, we found higher tumor recurrence rate ($P<0.001$) and shorter survival ($P<0.001$) existed in groups with increasing factors.

CONCLUSION: We first provide the prognostic value and linkage of three molecular markers in GISTs. The combination of three factors (p53, tumor size, and tumor mitosis) provides a more powerful prediction of prognosis than any single factor does.

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Keywords: Gastrointestinal stromal tumor; GIST; p53; PCNA; Ki-67; Prognosis

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Abstract

AIM: To elucidate the prognostic role and relationship of three molecular markers such as tumor suppressor gene p53, proliferating cell nuclear antigen (PCNA) and Ki-67 in gastric stromal tumor.

METHODS: A total of 108 surgically resected gastric smooth muscle tumor specimens were collected from January 1987 to December 1999. Immunohistochemical studies were performed on the paraffin sections of 99 of 108 CD117-positive tumors with antibodies of p53, PCNA, and Ki-67. Immunoreactivity of three molecular markers was recorded by labeling index (LI, %) and was analyzed for clinicopathologic and survival correlation.

RESULTS: Of the 99 cases, immunostaining revealed that 52 patients (52.5%) had p53, and 37 patients (37.3%) had Ki-67 immunoreactivity (defined as $>10\%$ of LI). All patients (100%) had PCNA immunoreactivity ranging from 12% to 93% of LI, divided into high or low by median. Statistics revealed that LI of three markers positively correlate to each other ($P<0.01$) and to microscopic tumor mitotic counts ($P<0.001$). By combination, patients with ≥ 2 markers (positive or high) in tumors had early tumor recurrence ($P<0.001$) and unfavorable outcome ($P<0.001$). Univariate analysis indicated that patients with tumor size >5 cm ($P=0.003$), tumor mitosis $>5/50$ HPF ($P<0.001$), p53 immunoreactivity ($P=0.001$), Ki-67

INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are interstitial tumors arising in the wall of the gastrointestinal tract. The term, GIST was first introduced in the 1980s to include a group of non-lymphomatous, non-epithelial tumors of the gut^[1]. GISTs have strong morphological similarities in their pathological features, but have heterogeneous immunophenotype and biological behavior^[2]. In the past, spindle cell tumors of the gastrointestinal (GI) tract were usually classified as smooth muscle tumors (leiomyomas, or leiomyosarcomas). Over the past two decades, advances in pathology and molecular genetics have provided further evidences regarding the interstitial cells of Cajal as the progenitor cells for GIST^[3,4]. The use of c-kit receptor tyrosine kinase antibodies of KIT with a panel of other antibodies may distinguish true GIST from smooth muscle

Table 1 Sources of antibodies and ratio of dilutions used in this study

| Antibody | Source | Dilution |
|-----------------------------------|---|----------|
| CD117 monoclonal | Dako M7140 Clone104D2 | 1:50 |
| CD 34 monoclonal | Dako M7161 CloneQBEnd10 | 1:200 |
| Desmin monoclonal | Novocastra NCL-DES-DERII Clone DE-R-11 | 1:100 |
| Smooth muscle actin monoclonal | Novocastra NCL-SMA Clone asm | 1:50 |
| S-100 polyclonal | Novocastra NCL-S100p | 1:1 500 |
| P53 monoclonal | Dako | 1:200 |
| Ki67 monoclonal | Dako MIB1 | 1:50 |
| PCNA monoclonal | Dako M0879 | 1:50 |

tumors, neural tumors, and other spindle cell neoplasms^[5,7]. Thereafter, the gastric and intestinal smooth muscle tumors in the earlier data largely would be classified as GISTs by immunohistochemical methods^[5,7].

Concerning prognosis, many studies have established clinicopathologic correlations, yet the criteria claimed to predict the prognosis remains vague. Although the proliferating activity in terms of the mitotic count is generally accepted as a prognostic indicator^[1,8,10], other gross and histologic parameters have been reported to influence the course of disease, such as tumor size^[9-11], cellular density^[11,12], predominant cell type^[13,14], neural or muscular component^[13,15], necrosis^[10,12] and histological grades^[10,14,16]. It has been suggested that in addition to the use of KIT expression in the diagnosis of GIST, c-kit mutation may also correlate with the malignant potential of GIST^[17-19]. Molecular markers by immunohistochemical studies dealing with proliferating index like Ki-67^[20-23], proliferating cell nuclear antigen (PCNA)^[8,24,25], and tumor suppressor gene p53^[20,22,26], have been applied with encouraging results. However, the reports reflect a considerable controversy in considering the different conclusions of prognostic role in these molecular markers. Further, there were less linkage and elucidation of correlation between these genes in the previous literatures. Besides, there was a lack of documentation of KIT positive stromal tumor in the early reports that the conclusion might be misleading.

In our previous study of 81 gastric stromal tumors in the Taiwanese^[9], we have found that mitotic numbers and tumor sizes were two independent prognostic factors for disease free survival of patients. The conclusion was comparative to other studies. With the current investigation, we first elucidate the prognostic role of 99-CD117 (+) gastric stromal tumors by combination of three molecular markers such as tumor suppressor gene p53, PCNA and Ki-67. Their expression and correlation in GIST will be further verified in this original article.

MATERIALS AND METHODS

Subjects

A total of 108 surgically resected gastric smooth muscle tumor specimens were collected at Kaohsiung Chang-gung Memorial Hospital from January 1986 to December 1999. Patients with tumors which could not be completely

resected or patients with evident metastasis on diagnosis were excluded. All the tumors were obtained from curative resection and were diagnosed as leiomyoma or leiomyosarcoma by pathological studies. The diagnosis of malignant tumor was based on the index of mitosis as follows: benign < 5 and malignant $\geq 5/50$ high-power fields (HPF, single field area of 0.20 mm²). The closing date of follow-up was December 31, 2004. Patients who died due to post-surgical complications were excluded from the study. Data on age, gender, and follow up duration were collected. In each case, all slides were reviewed, and the following histological parameters were regarded and recorded by two pathologists: 1) cellularity (low, medium or high); 2) predominant cell type (spindle, epithelioid or mixed); 3) nuclear pleomorphism (mild, moderate or severe). Cell type was categorized as being predominantly spindle (>75% of the tumor), epithelioid (>75% of the tumor), or mixed if both the spindle and epithelioid components occupied >25% of the tumor. Nuclear pleomorphism, defined as variation in nuclear size and shape, was judged to be mild, moderate or severe, with the highest level of nuclear pleomorphism recorded in each tumor. The tumor locations were classified into three groups as antrum, body and fundi-cardiac areas.

Immunohistochemistry

Tissue specimens were maintained in formaldehyde-fixed, paraffin-embedded blocks. Sections stained with hematoxylin and eosin (HE) were also reviewed. The paraffin sections from the specimens were deparaffinized, blocked with 30 mL/L hydrogen peroxide for 10 min, and subjected to antigen retrieval with microwave in 0.01 mol/L citrate buffer for 15 min. The slides were then washed twice with PBS, incubated with primary antibodies of CD117, CD34, smooth muscle actin (SMA), S-100, desmin, Ki-67, p53 and PCNA for 30 min each, then examined with a peroxidase conjugate using polymer detection system (Zymed Cat. No. 87-89431) for 30 min. Specific details of the immunohistochemical condition used for each are shown in Table 1. The antibody staining was visualized with 3,3-diaminobenzidine tetrahydrochloride (DAB; Sigma, St. Louis, MO, USA) in 0.1 mol/L Tris pH 7.2, containing 0.1 mL/L H₂O₂. The section slides were counterstained with Gill's hematoxylin, dehydrated, and mounted. Control tissues included mastocytoma of skin for positive control, and gastric carcinoma tissues and CD117 spared GIST for negative controls.

The markers (CD117, CD34, SMA, desmin, and S100) in the tumor cells on each slide were expressed as negative or as positive stain by estimating the number of positive tumor cells in the staining intensity. Negative was defined as no staining, or less than 10% of area with positive staining. Positive staining was defined as full or numerous staining. The labeling index (LI) of Ki-67, p53 and PCNA was calculated by two pathologists for each case. The percentage of Ki-67, p53, and PCNA-labeled tumor cells (from counting 1 000 cells) was calculated thrice for each tumor and an average of the three counts used for subsequent analyses. There was disagreement between proportion scores given by the two assessors in less than 5% of the cases for each immunostain. Any cases with

Table 2 The correlation between labeling index of molecular markers and pathologic parameters

| | Tumor mitosis | Tumor size | P53 | Ki67 | PCNA |
|---------------|--|--|--|--|--|
| Tumor mitosis | | CC=0.319 <i>P</i> =0.001 ¹ | CC=0.488 <i>P</i> <0.001 ¹ | CC=0.394 <i>P</i> <0.001 ¹ | CC=0.657 <i>P</i> <0.001 ¹ |
| Tumor size | CC=0.319 <i>P</i> =0.001 ¹ | | CC=0.285 <i>P</i> =0.004 ¹ | CC=0.032 <i>P</i> =0.751 | CC=0.206 <i>P</i> =0.041 ¹ |
| P53 | CC=0.488 <i>P</i> <0.001 ¹ | CC=0.285 <i>P</i> =0.004 ¹ | | CC=0.434 <i>P</i> <0.001 ¹ | CC=0.508 <i>P</i> <0.001 ¹ |
| Ki67 | CC=0.394 <i>P</i> <0.001 ¹ | CC=0.032 <i>P</i> =0.751 | CC=0.434 <i>P</i> <0.001 ¹ | | CC=0.295 <i>P</i> =0.003 ¹ |
| PCNA | CC=0.657 <i>P</i> <0.001 ¹ | CC=0.206 <i>P</i> =0.041 ¹ | CC=0.508 <i>P</i> <0.001 ¹ | CC=0.295 <i>P</i> =0.003 ¹ | |

CC: correlation coefficient. ¹Correlation is significant at the 0.05 level (two-tailed).

discrepant scores were reassessed to produce final scores for further analyses. The LI of Ki-67 and p53 more than 10% was defined as “positive” staining *vs* “negative” as less than 10%. Immunostaining of PCNA was recorded by percentage and was divided into two groups (by median) as high or low expression for further comparison.

Statistical analysis

The ages and tumor sizes were expressed as mean±SD. Comparisons between groups of independent samples were assessed by the Student's *t* test. Associations between categorical variables were assessed using χ^2 test. The correlation between continuous variables was determined by Spearman's rank correlation test. Survival rates were calculated by the Kaplan-Meier method and the difference in survival was compared with the log rank test. The influence of various clinicopathological features on survival was assessed by the Cox's proportional hazard model. A *P* value less than 0.05 was considered statistically significant.

RESULTS

Of these 108 patients, nine had tumors lacking immunoreactivity for c-kit. These c-kit negative tumors were more likely to show positivity for smooth muscle markers or for S100 than the 99 c-kit positive tumors. Only one of the patients with c-kit negative tumors had tumor recurrence and died. The other eight patients had no disease recurrence at the end of follow-up. Finally, these nine patients were excluded for the study population of molecular markers. A total 99 cases of GISTs were included comprising 40 males and 59 females, with age ranging from 31 to 80 (mean: 58±9.7) years and tumor size ranging from 1 to 26 (mean 6.8±4.2) cm. There were 61 benign and 38 malignant tumors based on the microscopic mitotic activities. Tumors were located in the fundi-cardiac region in 52 cases, body area in 37 cases and antral area in 10 cases.

The median follow-up period was 60 (range 3-160) mo. At the end of follow-up, 24 of 99 patients (24.2%) had disease recurrence, and 23 patients died of tumors. Subsequent survival analyses revealed that patients with smaller tumor size, less mitotic activity, and female gender had lower recurrence rates (*P*<0.05) and longer survival

(*P*<0.05) than patients with larger tumor size, more mitotic activity, and male gender (Figures 1A-1C; Tables 3 and 4). The 1-, 3-, and 5-year disease free survival rates were 100%, 93%, 93% (tumor <5 cm); 93%, 86%, 80% (5 ≤ tumor <10 cm); and 55%, 30%, 30% (tumor ≥10 cm), respectively. In addition, The 1-, 3-, and 5-year disease free survival rates were 98%, 89%, 89% (mitosis <5/50 HPF); 84%, 84%, 84% (5 ≤ mitosis <10/50 HPF); and 68%, 42%, 33% (mitosis ≥10/50 HPF), respectively. Moreover, there was strong positive correlation between tumor sizes and tumor mitotic counts (*P*=0.001) (Table 2).

Subsequently, the results of immunostaining for p53, Ki-67 and PCNA were analyzed. The immunoreactivity of these markers was calculated by labeling index (LI, %). The results revealed that 52 patients (52.5%) had positive p53, and 37 patients (37.3%) had positive Ki-67 immunoreactivity (defined as more than 10%, Figure 2). All patients had positive PCNA immunoreactivity, ranging from 12% to 93%, with a median of 70% and mean of 66% (Figure 2). Statistical analyses revealed that the LI of three markers strongly correlate to each other. The *P* value was less than 0.001 for p53 *vs* PCNA; less than 0.001 for p53 *vs* Ki-67; and 0.003 for Ki-67 *vs* PCNA, respectively (Table 2). Furthermore, we also found that LI of all three markers strongly correlate to microscopic tumor mitotic counts (all *P*<0.001). But only LI of p53 (*P*<0.001) and PCNA (*P*=0.04) correlate to tumor sizes (Table 2). Besides that, p53 positivity also positively correlates to CD34 positivity (*P*=0.001), and high PCNA positively correlates to nuclear pleomorphism (*P*=0.008).

Univariate survival analysis revealed that patients with larger tumor size (≥5 cm, *P*=0.003), more tumor mitosis (≥5/50 HPE, *P*<0.001), positive p53 immunoreactivity (*P*=0.001), positive Ki-67 immunoreactivity (*P*=0.026), high PCNA LI (above median, *P*=0.015) and male gender (*P*=0.036) were six predictors for earlier disease recurrence (Figure 1, Table 3). In addition, these six factors also predict overall survival of patient, as tumor size (*P*=0.004), tumor mitosis (*P*<0.001), p53 immunoreactivity (*P*=0.001), Ki-67 immunoreactivity (*P*=0.048), PCNA LI (*P*=0.025), and male gender (*P*=0.021) (Table 4).

Further multivariate analysis by Cox's proportional hazard model revealed that only mitotic number, tumor size, and p53 immunoreactivity were independent prog-

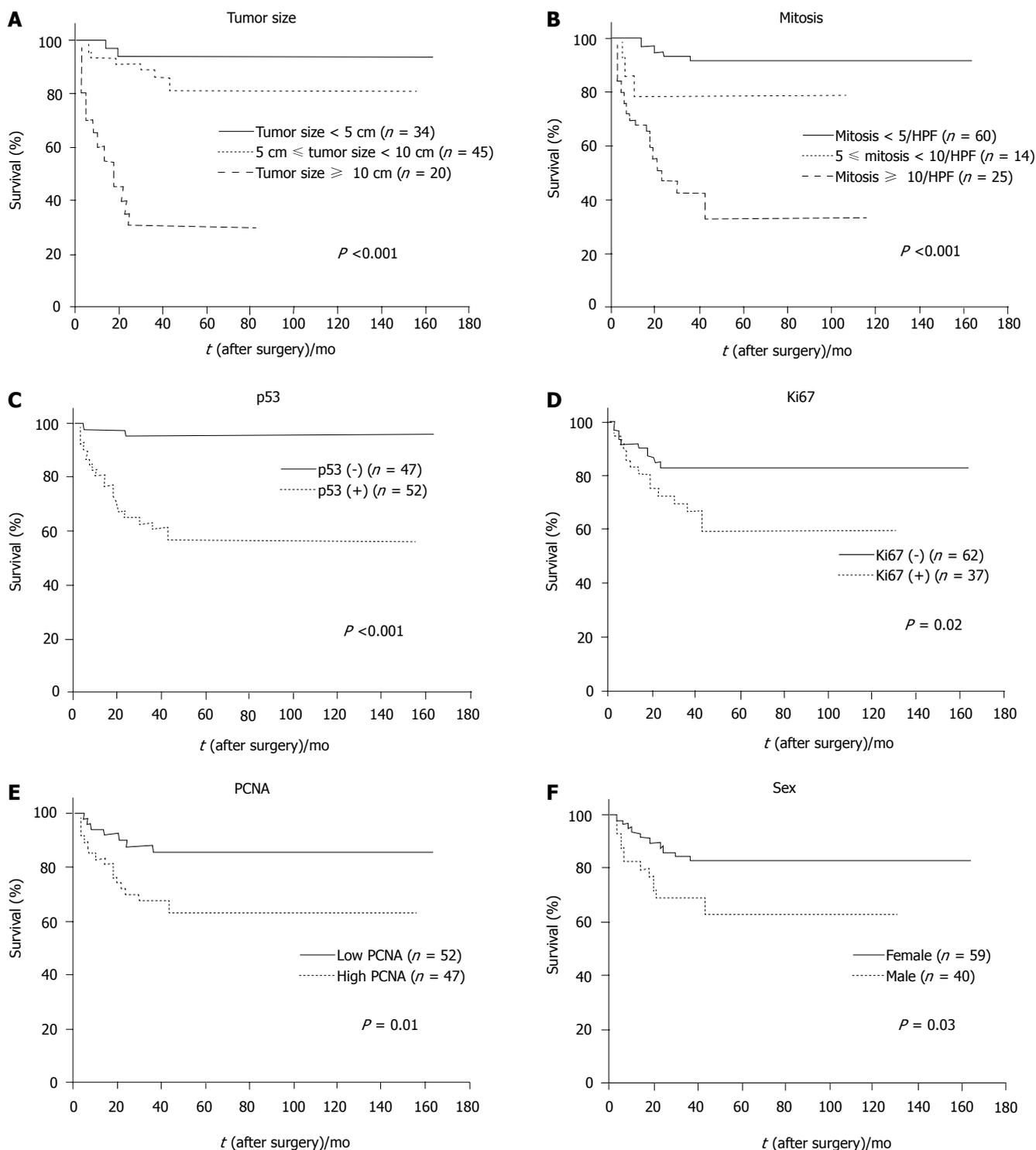


Figure 1 Kaplan-Meier analyses for patients survival (log rank test).

nostic factors for disease free and overall survival of GIST patients (Tables 3, 4). If we combine three molecular markers (p53 and Ki-67 positivity and high PCNA LI) for comparison, patients with more than two positive/high markers in their tumors had early tumor recurrence and unfavorable outcome (Figure 3A). Subsequently, we further combined three independent prognostic factors (p53 positivity; tumor size ≥ 5 cm; and tumor mitosis $\geq 5/50$ HPF) and divided the patients into four subgroups as 1) none of the event present; 2) one of the three events

present; 3) two of three events present and 4) all three events present. Statistical analysis revealed earlier tumor recurrence ($P < 0.001$) and shorter survival ($P < 0.001$) were found in the groups with increasing events. The 1-, 3-, 5-year recurrence rates were 0%, 0%, 0% (Group 1); 3.2%, 9.7%, 9.7% (Group 2); 4.5%, 15%, 15% (Group 3); and 42%, 67%, 77% (Group 4), respectively (Figure 3B). Further, 1-, 3-, 5-year overall survival rates were 100%, 100%, 100% (Group 1); 100%, 96%, 93% (Group 2); 95%, 90%, 83% (Group 3); and 62%, 37%, 22% (Group 4),

Table 3 Univariate and multivariate analyses for disease free survival rates of individual parameters of GIST patients

| | Univariate | | | Multivariate | | |
|---|------------|-----------|-----------------------|--------------|-----------|--------------------|
| | Risk | 95%CI | P | Risk | 95%CI | P |
| Age ≥ 60 / <60 yr | 1.01 | 0.45-2.25 | 0.98 | - | - | - |
| Gender M/F | 2.38 | 1.05-5.36 | 0.036 ^a | - | - | 0.1 |
| Tumor size ≥ 5 / <5 cm | 6.16 | 1.83-20.6 | 0.003 ^a | 3.43 | 1.12-11.8 | 0.025 ^a |
| Mitoses ≥ 5 / <5 /50 HPF | 6.30 | 2.49-15.9 | <0.001 ^a | 3.28 | 1.25-8.59 | 0.009 ^a |
| Tumor location fundic-cardiac/body-antrum | 0.99 | 0.54-1.81 | 0.98 | - | - | - |
| Hemoglobin ≥ 100 / <100 | 2.21 | 0.86-5.63 | 0.095 | - | - | - |
| Cell type spindle/non-spindle | 1.23 | 0.73-2.08 | 0.42 | - | - | - |
| Cellularity low+intermediate/high | 1.26 | 0.82-2.16 | 0.38 | - | - | - |
| Pleomorphism +/- | 0.82 | 0.36-1.89 | 0.65 | - | - | - |
| P53 +/- | 12.1 | 2.83-51.3 | 0.001 ^a | 6.45 | 1.45-28.7 | 0.002 ^a |
| Ki67 +/- | 2.52 | 1.12-5.68 | 0.026 ^a | - | - | 0.41 |
| PCNA above or below median | 2.97 | 1.23-7.17 | 0.015 ^a | - | - | 0.42 |
| CD 34 +/- | 3.07 | 0.41-22.7 | 0.27 | - | - | - |
| SMA +/- | 1.34 | 0.53-3.37 | 0.54 | - | - | - |
| Desmin +/- | 0.74 | 0.17-3.16 | 0.68 | - | - | - |
| S-100 +/- | 2.19 | 0.65-1.37 | 0.20 | - | - | - |

^a $P < 0.05$ vs others.

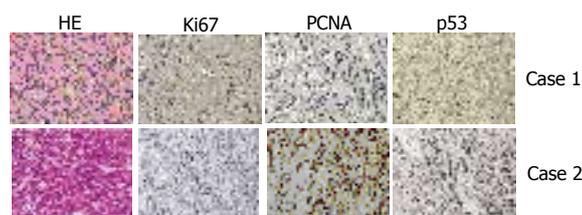


Figure 2 Immunohistochemical studies of p53, Ki67 and PCNA. Case 1 Negative p53, Ki-67 and low PCNA. Case 2 Positive p53, Ki-67 and high PCNA.

respectively. The combination of three factors provides a more powerful prediction of prognosis than any single factor does.

DISCUSSION

Several recent studies have suggested that tumor size and mitotic count, alone or in combination^[27,28], as well as Ki-67^[20,23-28] or PCNA^[8,24,25] proliferative index, and tumor suppressor gene p53^[20,22,26] are useful in predicting the clinical behavior of GIST. However, the varying conclusions drawn from previous studies are matched by varying deficiencies of these studies. First, relatively small case numbers were studied. Second, prognostic studies should not pool GISTs from different locations into a single heterogeneous group, but evaluate prognostic factors relating to each homogeneous tumor site. Moreover, previously published series^[11,28,29] indicated that distally located GISTs (small intestine, colon) seem to behave in more aggressive patterns than gastric tumors. Third, many studies were done before KIT detection was available. The distinction between GIST and pure neural or smooth muscle tumors is important as the latter may portend a better prognosis^[27,30]. Fourth, the reports reflect a considerable controversy in considering the different conclusions of prognostic role in these molecular markers.

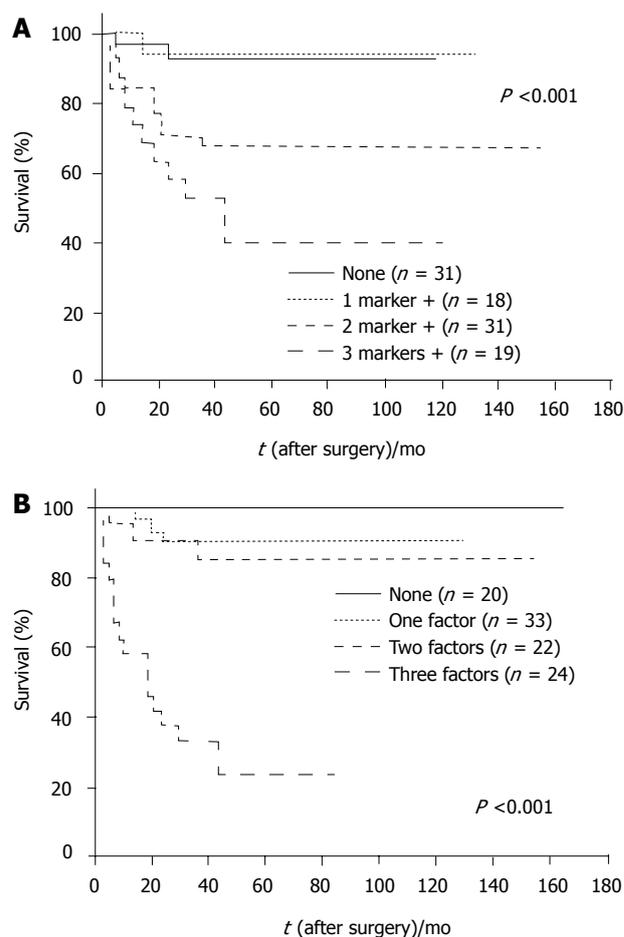


Figure 3 Combination of molecular markers and prognostic factors **A**: p53 and Ki-67 positivity and high PCNA LI for survival analysis (log rank test) prognostic factors **B**: p53 positivity; tumor size ≥ 5 cm; and tumor mitosis $\geq 5/50$ HPF.

In addition, there was also less linkage and elucidation of correlation between these genes in the previous literatures.

To overcome these deficiencies, we conducted a large

Table 4 Univariate and multivariate analyses for overall survival rates of individual parameters of GIST patients

| | Univariate | | | Multivariate | | |
|---|------------|------------|--------------------|--------------|-----------|--------------------|
| | Risk | 95%CI | P | Risk | 95%CI | P |
| Age ≥ 60 / <60 yr | 0.90 | 0.39-2.06 | 0.81 | - | - | - |
| Gender M/F | 2.68 | 1.16-6.20 | 0.021 ^a | - | - | 0.097 |
| Tumor size ≥ 5 / <5 cm | 19.01 | 2.56-141.1 | 0.004 ^a | 10.17 | 1.34-76.9 | 0.001 ^a |
| Mitoses ≥ 5 / <5 /50 HPF | 10.30 | 3.48-30.4 | $<0.001^a$ | 5.22 | 1.70-15.9 | 0.001 ^a |
| Tumor location fundic-cardiac/body-antrum | 1.07 | 0.58-1.96 | 0.81 | - | - | - |
| Hemoglobin ≥ 100 / <100 | 1.98 | 0.78-5.05 | 0.14 | - | - | - |
| Cell type spindle/non-spindle | 1.29 | 0.76-2.18 | 0.34 | - | - | - |
| Cellularity low+intermediate/high | 1.21 | 0.64-2.17 | 0.35 | - | - | - |
| Pleomorphism +/- | 0.90 | 0.39-2.09 | 0.82 | - | - | - |
| P53 +/- | 11.29 | 2.64-48.2 | 0.001 ^a | 4.84 | 1.08-21.6 | 0.012 ^a |
| Ki67 +/- | 2.30 | 1.01-5.26 | 0.048 ^a | - | - | 0.70 |
| PCNA above or below median | 2.75 | 1.13-6.70 | 0.025 ^a | - | - | 0.74 |
| CD 34 +/- | 1.98 | 0.58-6.70 | 0.27 | - | - | - |
| SMA +/- | 1.38 | 0.54-3.52 | 0.48 | - | - | - |
| Desmin +/- | 0.76 | 0.18-3.27 | 0.72 | - | - | - |
| S-100 +/- | 2.28 | 0.67-7.70 | 0.18 | - | - | - |

cases review of 99 KIT-positive GISTs which were limited at the gastric area. By combination of these molecular markers and other clinicopathological factors, these results might shed a light to elucidate the clinical behavior of GIST. In the present study, we provided prognostic evidence of three molecular markers Ki-67, PCNA and p53 in GISTs. The expression of single gene closely correlates to each other. Further, each single gene had diagnostic value of malignant potential (correlation to tumor mitosis) of GIST. These findings suggested that high proliferative states as well as p53 positivity occur in a portion of GISTs and may constitute poor prognosis. The expression of tumor suppressor gene p53 correlated the proliferative states (PCNA, Ki-67, tumor mitosis), tumor sizes, and CD 34 positivity. Among these factors, p53 positivity, tumor mitosis, and tumor sizes were three independent prognostic factors. By combination of three independent prognostic factors, it provided the more powerful prediction for disease recurrence and survival of GIST patients than any individual factor did.

PCNA is a nuclear protein, which is closely related to the cell cycle regulation being an auxiliary molecule for DNA polymerase- δ ^[31]. It is also an auxiliary protein present during G₁-late phase and S phase. P53, a tumor suppressor gene, has an important function in DNA repair and in regulation of apoptosis. Mutations of p53 were described in malignant tumors and can be the cause of the alterations of this balance. The correlation between p53 and PCNA expression in other malignancies has been reported^[32,33]. Progression of neoplasia is often associated with the loss of cell cycle control. PCNA overexpression might predict more aggressive tumor behavior. In general, p53 positivity correlated with increased PCNA labeling index in neoplasia and represented advanced disease states and poor outcome. However, their correlation in GIST was not reported. In the present study, we first found that there was strong correlation between p53 and PCNA index in GIST, which was comparative with previous reports of other malignancies. It might be explained that the cyclin-

dependent kinase inhibitor p21/WAF1 is regulated by p53-dependent and p53-independent pathways. In addition, p21/WAF1 binds with PCNA and inhibits the action of PCNA^[34]. Overexpression of p53 on immunostaining (mostly p53 mutation) might disturb this pathway and trigger PCNA activity, thereby promoting cancer cells proliferation.

Of the three supplementary immunohistochemical markers tested, Ki-67 was most widely studied^[20-23,28] and it was also demonstrated in other malignancies^[35]. Ki-67 protein was considered to be more specific and reliable than PCNA as a marker of cell proliferation^[28]. However, two other studies reached significant conclusion by using PCNA instead of Ki-67 in GISTs^[24,36]. But they were all studied before KIT protein was available to detect GIST. In addition, whether Ki-67 or PCNA index is more practical and reliable than mitotic counting is still a matter of ongoing controversy. It has been suggested that there was less interobserver variation for Ki-67 index than for mitotic counting^[37]. But there was no comprehensive report dealing with the comparisons with PCNA. In the present study, the mitotic counting and immunostaining reading were performed by two pathologists to avoid interobserver bias. Based on the large case number of KIT-positive GISTs, our finding suggested that mitotic counting was still superior to Ki-67 or PCNA to predict prognosis in gastric GIST, even though they are closely related to each other. Although both Ki-67 and PCNA predict disease recurrence and overall survival of patients after the operation, they are not independent prognostic factors. Therefore, we are unable to demonstrate the priority of clinical application between Ki-67 and PCNA.

In studies of gastrointestinal smooth muscle tumors/GISTs, p53 positivity was a negative prognostic marker^[38,39]. However, p53 expression was prognostic in other studies^[20,40]. In our series, we found that p53 labeling index strongly related to three mitotic indices (Ki-67, PCNA, and tumor mitotic count) and represented an independent prognostic role in GIST patients. There

were several possible reasons for the discrepancy between these studies. First, different definition of positivity of p53 expression and different intensity of immunostaining read. Second, different dilutions and antigen retrieval methods were used. Third, whether disease related deaths were used as endpoints for survival analysis. To overcome this, we adopted a multifactorial method to evaluate the patients' prognosis. By combination of three molecular markers or three independent factors, the prediction of prognosis is more powerful than any single factor did. It might provide a further insight to deal with the clinical behavior of GIST in future. In conclusion, we demonstrated the first evidence of linkage between two proliferating markers and one tumor suppressor gene p53 in GISTs and the relationship between these markers and pathological mitotic counts and tumor size. By combination of these factors, it provided a more exact and powerful prediction of the prognosis of GIST patients after surgery.

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Pilot study of postoperative adjuvant chemoradiation for advanced gastric cancer: Adjuvant 5-FU/cisplatin and chemoradiation with capecitabine

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toxicity profile to the chemoradiation regimen utilized in INT-0116. This treatment modality allowed successful loco-regional control rate and 3-year overall survival.

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Keywords: Gastric cancer; Postoperative; Adjuvant chemotherapy; Chemoradiation

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Abstract

AIM: To evaluate the efficacy and toxicity of postoperative chemoradiation using FP chemotherapy and oral capecitabine during radiation for advanced gastric cancer following curative resection.

METHODS: Thirty-one patients who had underwent a potentially curative resection for Stage III and IV (M0) gastric cancer were enrolled. Therapy consists of one cycle of FP (continuous infusion of 5-FU 1000 mg/m² on d 1 to 5 and cisplatin 60 mg/m² on d 1) followed by 4500 cGy (180 cGy/d) with capecitabine (1650 mg/m² daily throughout radiotherapy). Four wk after completion of the radiotherapy, patients received three additional cycles of FP every three wk. The median follow-up duration was 22.2 mo.

RESULTS: The 3-year disease free and overall survival in this study were 82.7% and 83.4%, respectively. Four patients (12.9%) showed relapse during follow-up. Eight patients did not complete all planned adjuvant therapy. Grade 3/4 toxicities included neutropenia in 50.2%, anemia in 12.9%, thrombocytopenia in 3.2% and nausea/vomiting in 3.2%. Neither grade 3/4 hand foot syndrome nor treatment related febrile neutropenia or death were observed.

CONCLUSION: These preliminary results suggest that this postoperative adjuvant chemoradiation regimen of FP before and after capecitabine and concurrent radiotherapy appears well tolerated and offers a comparable

INTRODUCTION

Numerous trials of postoperative chemotherapy or immunotherapy for advanced gastric cancer have shown no conclusive benefits^[1,2]. The use of adjuvant radiation therapy in the treatment of gastric cancer has been investigated owing to the substantial incidence of local and regional failure after primary surgical resection. Following a potentially curative resection of the gastric cancer, a United States Intergroup study (INT-0116) demonstrated superior survival for patients who received a chemoradiotherapy^[3]. These results established a new standard care for patients following curative resection of gastric cancer. However, the principal benefit associated with postoperative 5-FU/leucovorin (LV), and radiotherapy was reduction of the loco-regional failure; differences in the rate of distant failure were less pronounced.

We conducted a pilot study to examine the efficacy of chemotherapeutic regimens FP (Cisplatin and continuous infusion of 5-FU) that replaced 5-FU/LV and capecitabine for the radiosensitizer during external radiotherapy in patients with advanced gastric cancer.

MATERIALS AND METHODS

Patients and evaluation

The study population was composed of 31 patients with

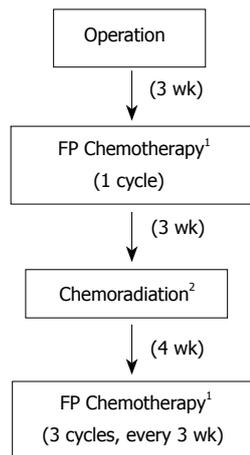


Figure 1 Schema for postoperative chemoradiation study. ¹FP Chemotherapy: 5-FU 1000 mg/m² continuous infusion on d 1-5, cisplatin 60 mg/m² on d 1. ²Chemoradiation: 4500 cGy at 180 cGy/d with capecitabine 1650 mg/m² daily during the entire radiotherapy course.

advanced gastric cancer who underwent a potentially curative gastrectomy with D2 lymph node dissection at Dong-A University Medical Center between March 2002 and September 2004. Eligibility criteria included histologically confirmed gastric adenocarcinoma, defined as resection performed with curative intent with negative pathologic margin, no gross or microscopic residual disease; disease stage IIIA to IV (M0) by the staging criteria of the American Joint Commission on Cancer (AJCC, 6th edition)^[4]. All patients had undergone extensive (D2) lymph node dissection. Other requirements were as follows; hemoglobin level ≥ 100 g/L, normal white blood cell and platelet count, normal kidney/liver function, Eastern Cooperative Oncology Group (ECOG) performance status of ≤ 1 ^[5], a caloric intake > 1500 kcal per day by oral route and beginning of treatment within 4 wk of surgery. This study protocol was reviewed and approved by the Dong-A University Medical Center Institutional Review Board.

Chemoradiotherapy

Three wk after surgery, all patients received one cycle of FP chemotherapy (5-FU 1000 mg/m² continuous infusion on day 1-5, cisplatin 60 mg/m² on d 1) followed by regional radiotherapy with capecitabine (1650 mg/m² daily during the entire radiotherapy course) in three wk. Four wk after completion of radiotherapy, patients received three additional cycles of FP regimen every three wk (Figure 1). According to phase I trial evaluating the concurrent combination of radiotherapy and capecitabine in rectal cancer^[6], oral capecitabine was given at a dose of 1650 mg/m² daily, which was divided into two doses given 12 h apart. A total dose of 4500 cGy in 25 fractions over five wk was delivered to the target volume that included the gastric bed, anastomosis, stump, and regional lymph nodes areas. We used the definition of the Japanese Research Society for Gastric Cancer for the delineation of the regional node areas^[7]. The tumor bed was defined by preoperative computed tomographic (CT) imaging, and in some instances, surgical clips^[8]. Radiation was delivered with 15 MV photons. Typical anterior-posterior/posterior-anterior (AP-PA)

Table 1 Characteristics of patients (*n* = 31)

| Characteristics | Number of (%) patients |
|------------------------------|------------------------|
| Age (yr) | |
| Median | 51.9 |
| Range | 24-70 |
| Sex | |
| Male | 21 (67.8) |
| Female | 10 (32.2) |
| Type of operation | |
| Radical subtotal gastrectomy | 16 (51.6) |
| Radical total gastrectomy | 15(48.4) |
| Location | |
| Antrum | 17 (54.8) |
| Corpus | 9 (29.0) |
| Cardia | 5 (16.1) |
| Lauren classification | |
| Diffuse | 20 (64.5) |
| Intestinal | 6 (19.4) |
| Mixed | 5 (16.1) |
| Borrmann type | |
| I | 1 (3.2) |
| II | 4 (12.9) |
| III | 18 (58.1) |
| IV | 8 (25.8) |
| Stage | |
| IIIA | 11 (35.5) |
| T2bN2 | 2 (6.5) |
| T3N1 | 9 (29.0) |
| IIIB | 14 (45.2) |
| T3N2 | 14 (45.2) |
| IV (M0) | 6 (19.4) |
| T4N2 | 1 (3.2) |
| T2bN3 | 1 (3.2) |
| T3N3 | 4 (13.0) |
| Number of positive nodes | |
| Median | 9 |
| Range | 1-49 |

portals extended from the hemidiaphragm to the L3 body, and laterally, from the external border of the postoperative gastric remnant to include the whole gastric area, duodenum, and porta hepatis. Doses were limited so that less than 3000 cGy of radiation of the hepatic volume. The equivalent of at least two thirds of one kidney was spared from the field of radiation.

Outcome analysis

All patients were followed up every 3 mo for the first year and then every 4-6 mo for at least 3 years. The regular follow-up program entailed a physical examination, chest radiography, complete blood count, liver function tests, abdominopelvic computed tomography and gastroscopy as clinically indicated. Toxicities were recorded according to the National Cancer Institute Common Toxicity Criteria^[9]. Disease relapse or progression was documented by biopsy or cytology reports or by clinical evidence of radiographic studies. Survival, as well as loco-regional and distant relapse, was analyzed with the Kaplan-Meier product limited

Table 2 Reasons for the cessation of chemoradiotherapy (*n* = 8)

| Reasons for cessation | Number of patients (%) |
|--|------------------------|
| Protocol treatment completed | 23 (74.2) |
| Toxic effects during chemoradiotherapy | 6 (19.3) |
| during maintenance chemotherapy | 2 (6.4) |
| Patients declined further chemotherapy | 4 (12.9) |
| | 2 (6.4) |

Table 3 Major toxic effects¹ of chemoradiotherapy

| Type of toxic effects | Number of patients (%) |
|------------------------------------|------------------------|
| Hematologic anemia | 4 (12.9) |
| Neutropenia | 15 (50.2) |
| Thrombocytopenia | 1 (3.2) |
| Nausea/Vomiting | 1 (3.2) |
| Diarrhea | 1 (3.2) |
| Adhesive ileus requiring operation | 2 (6.4) |

¹ Major toxic effects were defined as those of grade 3/4.

method. The site and date of first relapse, and the cause and date of death were recorded. The sites of relapse were classified as follows: the relapse was coded as loco-regional if tumor was detected within the radiation fields (including gastric bed, remnant stomach, and surgical anastomosis); as peritoneal if tumor was detected in the peritoneal cavity; and as distant if the metastases were diagnosed outside the peritoneal cavity or there was liver metastasis.

RESULTS

Patients characteristics

This study included 31 patients (21 males and 10 females) with ages ranging from 24 to 70 years (median: 51 years). Follow-up of patients ranged from 8 to 36 mo (median: 22.2 mo). Sixteen patients (51.5%) underwent subtotal gastrectomy and most tumors were located in antrum or corpus. In all patients, a D2 lymphadenectomy was performed. The patients were at high risk for relapse; all patients had pathologically involved nodes; 9(29%) N1 nodes, 17(54.8%) N2 nodes and 5(16.2%) N3 nodes, and 93.5% had stage T3 or T4 (Table 1).

Treatment

Of the 31 patients assigned to this study, 23(74.2%) completed treatment as planned. Treatment was interrupted in 6 patients (19.3%) because of toxic effects. Two patients (6.4%) declined further treatment. All patients, including those who stopped treatment, were reviewed (Table 2).

Toxicity

Toxicities of grade 3/4 are summarized in Table 3. The most common toxicity was neutropenia (50.2%). Anemia (12.9%), thrombocytopenia, nausea and vomiting, diarrhea and abdominal pain were also detected, and symptomatic

Table 4 Sites of relapse (*n* = 8)¹

| Site | Number of patients with relapse (%) |
|---------------------------|-------------------------------------|
| Locoregional ² | 3 (9.7) |
| Peritoneal ³ | 2 (6.4) |
| Distant ⁴ | 3 (9.7) |

¹Patients relapsed at multiple sites, the total number of relapsed sites was greater than the number of relapsed patients (*n*=4).

²Tumor detected within the radiation fields.

³Tumor detected in the peritoneal cavity.

⁴Metastases were outside the peritoneal cavity (including liver).

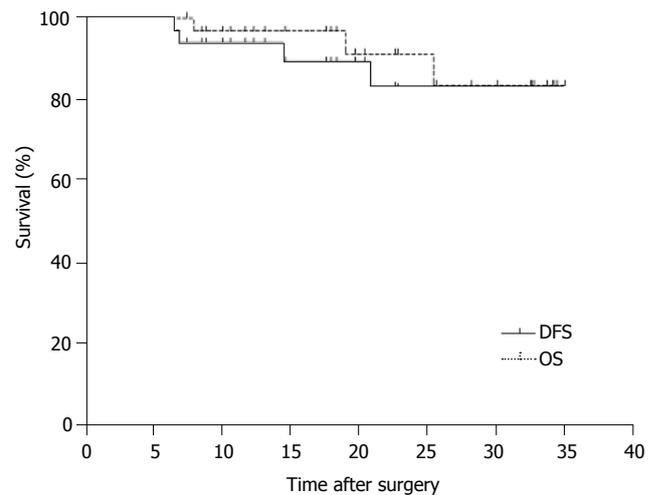


Figure 2 Overall survival and disease free survival for 31 patients with advanced gastric cancer treated with surgery followed by chemoradiotherapy.

treatments were performed as appropriate. There was no grade 3/4 hand foot syndrome, and there have been no treatment related febrile neutropenia. Most patients with grade 1/2 hand-foot syndrome were managed with vitamin B₆ administration and supportive care. Two patients (6.4%) underwent re-laparotomy for intestinal obstruction unrelated to tumor recurrence but of intestinal adhesion. No toxic death was observed.

Survival and relapse

During a median follow-up period of 22.2 mo, 3(9.7%) patients died of recurrence. The 3-year disease free and overall survivals in this study were 82.7% and 83.4%, respectively (Figure 2). Four patients (12.9%) were relapsed during follow-up. Loco-regional recurrence occurred in 3(9.7%) of the relapsed patients. Peritoneal relapse was reported in 2(6.4%) patients and 3(9.7%) had distant metastases. Patients had relapses at multiple sites, and the total number of relapse was greater than the numbers of relapsed patients (Table 4).

DISCUSSION

The major change in adjuvant therapy for gastric cancer comes from the results of the INT-0116 trial^[3], which used two cycles of 5-FU and leucovorin followed by concurrent chemoradiotherapy with 4500 cGy radiation given

using fields that encompassed the entire gastric bed. This study demonstrated a major advantage in overall survival, disease-free survival, and loco-regional control with the use of adjuvant chemoradiotherapy. These data suggest that the primary effect of postoperative chemoradiotherapy was a reduction in local and regional recurrence rates without apparent differences in distant relapse rates. However, questions have been raised about the adequacy of the chemotherapy and surgery of the study. The essential weakness of the INT-0116 study is that they performed D2 lymph node dissection in only 10% of patients. Hence, comparable local control might be achievable with optimal surgery that includes D2 lymph node dissection and the addition of more active chemotherapy.

The majority of trials using older systemic regimens for postoperative adjuvant chemotherapy for advanced gastric cancer failed to demonstrate a consistent reduction in the risk of recurrence^[1,2,10]. A modest but statistically significant survival benefit appeared to be confined to the Asian studies only in the postoperative chemotherapy group^[11-13]. The combination of cisplatin and 5-FU has also been extensively studied in gastric cancer. Phase II studies have indicated substantial activity with acceptable toxicity. Prospective randomized studies in patients with stage IV disease have not consistently demonstrated a significant improvement in survival for patients receiving cisplatin-containing regimens when compared to other combination or single agent^[10,14]. However, response rates have been in general higher when cisplatin was included. Therefore, there has been substantial interest in the use of cisplatin-containing regimens in the adjuvant setting.

Capecitabine is oral prodrug of 5-FU and it would be a reasonable alternative when 5-FU or biomodulated 5-FU alone is being considered. Interest has also been shown in the use of oral agents instead of continuous-infusion 5-FU. Capecitabine has generated a great deal of interest as a simpler method of drug delivery. It is absorbed intact through the gut and then activated by a series of enzymatic alterations^[15]. Phase II studies demonstrated that capecitabine had substantial activity in colorectal cancer, with an acceptable toxicity profile^[16]. Phase III randomized clinical trials performed both in the United States and Europe have now shown that this oral agent is at least as effective as intravenous 5-FU/leucovorin, and the toxicity profile of capecitabine is more favorable than that of the Mayo Clinic schedule of 5-FU^[17-19].

Compared with the results from postoperative chemoradiotherapy with D2 lymph node dissection study for gastric cancer^[20], if adjusted for stage, this study showed superior 3-year survival in patients with advanced gastric cancer. This treatment modality allowed chemoradiotherapy with minor toxicities. Although it is inconclusive regarding long-term influence of chemoradiotherapy using FP or capecitabine regimens as adjuvant setting in patients with advanced gastric cancer following curative resection, results of loco-regional control rate and 3-year survivals in this study are promising. This study adds to the limited data on the promising results of chemoradiotherapy in patients with advanced gastric cancer. Randomized clinical trials need to be mounted to critically evaluated adjuvant therapy strategies in advanced gastric cancer.

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RAPID COMMUNICATION

Computed tomography-guided transpulmonary radiofrequency ablation for hepatocellular carcinoma located in hepatic dome

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Abstract

AIM: To determine the feasibility of performing computed tomography (CT)-guided transpulmonary radiofrequency ablation (RFA) for hepatocellular carcinoma (HCC) located in the hepatic dome.

METHODS: A total of seven patients with HCC comprising seven nodules located in the hepatic dome were treated from April 2004 to December 2004. CT-guided transpulmonary RFA was performed using a cool-tip type electrode (Radionics Company) based on a standardized energy protocol. All tumors located in the hepatic dome were not detectable by the usual ultrasound (US) methods. The lesion diameters ranged from 15 to 27 mm.

RESULTS: RFA was technically feasible in all the patients. The puncture procedure was performed twice or less and the total average performance time was 40.6 min. Local tumor control was achieved in all the patients. The necrosis diameter ranged from 25 to 35 mm. The mean follow-up period was 9.6 (7-14 mo) mo. There was no local recurrence at the follow-up points. Pneumothorax requiring pleural drainage was the main complication, which was observed in two of the seven patients (28.6%). However, it improved with chest drainage tube, and the tube could be removed within 2-3 d. No other major complications were observed.

CONCLUSION: CT-guided puncture is useful for the treatment of tumors located in the hepatic dome which are hardly detectable by US, even though pneumothorax sometimes may occur as a complication. In the cases with adhesion in the pleura for which artificial pleural effusion methods are not appropriate, CT-guided RFA is thus considered to be an alternative treatment for HCC located in the hepatic dome.

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Key words: Radiofrequency ablation; Hepatocellular carcinoma; Interventional procedures; CT-guided transpulmonary RFA

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INTRODUCTION

Percutaneous radiofrequency ablation (RFA) is a standard therapy for hepatocellular carcinoma (HCC)^[1-4]. Ultrasound (US)-guided puncture is the simplest and most popular method for performing RFA treatment of HCC^[1-4]. However, depending on the location of tumors, it is often difficult to detect them by US^[5-7]. Especially, the subphrenic area is one of the most difficult places because the lung obstructs the ultrasound^[6,7]. As a result, some approaches using artificial pleural effusion method^[6-8], thoracoscopy^[9,10] or open surgery have been performed for HCC located in the hepatic dome. However, each approach has its advantages and disadvantages. The artificial pleural effusion method is sometimes complicated and requires a high level of skill for the performer. Furthermore, patients with adhesion of the pleura are not indicated for this procedure. A thoracoscopic approach or open surgery needs general anesthesia. Recently, RFA has been performed for the treatment of various neoplasms including lung cancer^[11,12]. Although pneumothorax is a major complication observed in 16% of all cases, lung RFA has shown itself to be a safe and feasible option for

Table 1 Patient characteristics and follow-up data of the study population

| Patients | Age/Sex | Preinterventional therapy | Child-Pugh classification | Number of tumor treated with transpulmonary RFA | Size (mm) | Location | Puncture procedure (times) | Puncture procedure (min) | Complication | Follow-up (mo) |
|----------|---------|---------------------------|---------------------------|---|-----------|----------|----------------------------|--------------------------|---|----------------|
| 1 | 60/M | TAE | A | 1 | 20 | S8 | 2 | 50 | Pneumothorax need the chest drainage tube | 12 |
| 2 | 71/M | TAE | A | 1 | 15 | S8 | 2 | 36 | Pneumothorax need the chest drainage tube | 9 |
| 3 | 77/F | TAE | A | 1 | 16 | S8 | 1 | 30 | No | 14 |
| 4 | 63/M | RFA | A | 1 | 27 | S8/4 | 2 | 55 | Small pneumothorax | 10 |
| 5 | 68/F | RFA | A | 1 | 15 | S8 | 1 | 34 | Small pneumothorax | 8 |
| 6 | 72/M | no | A | 1 | 15 | S8 | 1 | 40 | Small pneumothorax | 7 |
| 7 | 64/M | TAE | A | 1 | 22 | S8 | 2 | 39 | No | 7 |

the treatment of lung neoplasms^[11]. Shibata *et al.*^[13] have previously reported that CT-guided transpulmonary RFA is effective for eight patients with liver tumors in the hepatic dome. We have herein determined the utility of CT-guided transpulmonary RFA for the treatment of HCC located in the subphrenic area. The aim of this study was to assess the feasibility and efficacy of CT-guided transpulmonary RFA for HCC.

MATERIALS AND METHODS

Patients

A total of seven patients (five males, two females, mean age 67.9 ± 5.9 years) with HCC comprising seven nodules not indicative of undergoing usual US-guided RFA were included in this study and treated from April 2004 to December 2004. In four of these patients, transcatheter arterial embolization (TAE) for HCC was performed previously. In two patients, US-guided RFA was initially attempted but proved to be impossible to perform. The patients' characteristics are given in Table 1. RFA was performed after an interdisciplinary consensus conference, with the participation of hepatologists, radiologists, and surgeons. After detailed patient information was given, written consent was obtained. The inclusion criteria for RFA were a rejection of surgical therapy by the surgeon or the patient. The exclusion criteria were tumor size of 3 cm or more than three liver lesions, a prothrombin time $< 50\%$, and thrombocytes $< 50\,000/\mu\text{L}$. Tumors detectable with usual US methods were excluded. Within 2 wk prior to RFA, all patients underwent preinterventional CT imaging.

Radiofrequency ablation

After local anesthesia, a 17-gauge cooled-tip electrode with a 2- or 3 cm exposed tip (Radionics, Burlington, MA, USA) was attached to a radiofrequency generator and then inserted under CT guidance. The patient was placed in a supine position and two neutral electrodes were placed at the proximal thigh. For the localization of lesions, the skin entrance point was validated with a grid made from sealed plastic tubes. Thereafter, the cool-tip needle was inserted into the lesion. The punctures were controlled with CT imaging, which was acquired using the breath-hold technique. Finally, the position was checked

by CT imaging. After successful placement of the RFA needle, RFA was started. Both the temperature and tissue impedance were monitored during ablation. Cooled water was infused to maintain the tip temperature at $< 20^\circ\text{C}$. The radiofrequency energy was delivered for 6-12 min each time. For large lesions, the electrode was inserted into additional sites of the tumor nodule. After the ablation procedure was finished, CT was acquired with the RFA needle still in place to evaluate the size of necrosis and to rule out any remaining viable tumor parts in the region of ablation. Dynamic CT was then used to determine whether or not the tumor nodule was completely ablated and necrotized just after the procedure and a few days after RFA. For further follow-up, dynamic CT was performed after 1 mo and then every 3 mo after the procedure. Technical success was defined as complete tumor necrosis without any remaining contrast-enhanced tumor tissue a few days and 1 mo after the ablation procedure.

RESULTS

The cool-tip needle was successfully placed and RFA was successfully performed in all the patients. All the patients presented with interventional pain and were treated with a single dose of intravenous anesthesia. No further complications occurred during the intervention. The duration of the entire RFA procedure ranged 34-55 min (mean 40.6 min). The puncture procedure was performed twice or less and the average puncture time was 1.57 ± 0.53 times. The 1-mo follow-up examination showed a complete local tumor ablation in all the patients. The mean follow-up period was 9.6 (range 7-14 mo) mo. There was no local tumor recurrence in any patient. Two patients had intrahepatic recurrences and their treated lesions remained tumor-free. All patients survived 6 mo after the ablation. Five patients remained completely tumor-free during the follow-up period. The results are given in detail in Table 1 and a representative case is shown in Figure 1. Pneumothorax was noted in five cases (71.4%), including two cases (28.6%) of major pneumothorax requiring a chest tube. However, both of these patients improved due to chest drainage and the tube could be removed within 2-3 d. In addition, none of the patients demonstrated any clinical complications with problematic pleural effusion. Trans-pulmonary RFA showed itself to be a safe and

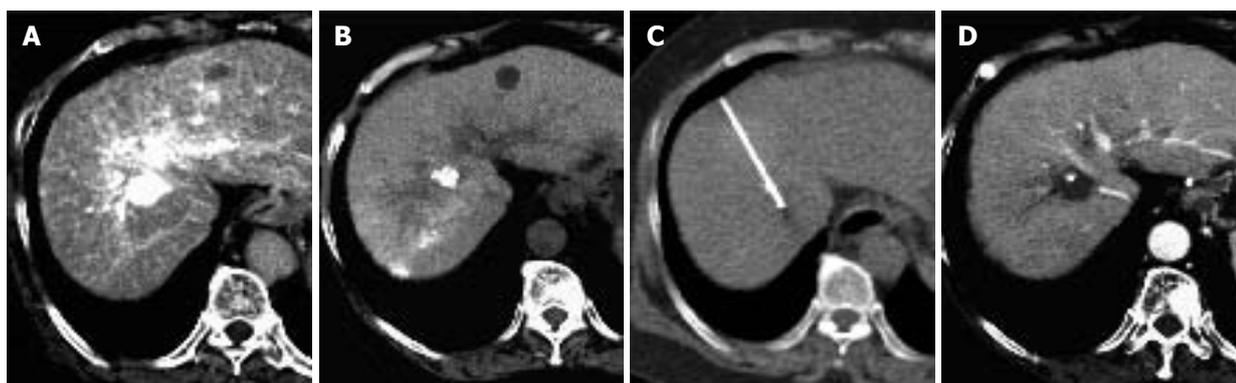


Figure 1 A 68-year-old female patient with hepatocellular carcinoma arising from hepatitis C-related liver cirrhosis. **A:** A hypervascular tumor measuring 15 mm in diameter in the subphrenic region by CT angiography; **B:** partially but insufficiently deposited lipiodol during post-transcatheter arterial embolization (TAE); **C:** location of the lipiodol deposition identified by CT-guided transpulmonary RFA; **D:** no local recurrence after 7 mo of RFA.

feasible option in the treatment of HCC located in the hepatic dome.

DISCUSSION

CT-guided transpulmonary RFA was successfully performed with a good local control for HCC by means of this relatively easy method in the present study. Percutaneous RFA is a standard therapy for HCC^[1-4]. One advantage of such percutaneous techniques is its repeatability in cases of local or distant tumor recurrence and its minimal invasive characteristics in comparison to surgery^[1-4]. Furthermore, RFA has been proved to be superior regarding the extent of tumor necrosis in comparison to percutaneous ethanol injection (PEI) for the treatment of small HCC^[14]. However, depending on the location of tumors, it is often difficult to detect them by US, especially in the subphrenic area. Minami *et al*^[6] have reported percutaneous US-guided RFA with the concurrent use of artificial pleural effusion for HCC located in the right subphrenic region. After 200-100 mL of 50 g/L glucose solution was infused intrathoracically to separate the lung and the liver, it became possible to obtain an image of the whole tumor, suggesting that US-guided RFA with artificial pleural effusion for HCC is safe and has no major complications. Although US-guided RFA with artificial pleural effusion is a good approach with minimal invasion^[6-8], its procedure is sometimes complicated and requires the performer with a high degree of experience. The success of US-guided RFA with artificial pleural effusion thus depends on the skill of the performer. In addition, some patients with adhesion of the pleura may not be indicated for artificial pleural effusion. CT-guided transpulmonary RFA does not require any infusion of the artificial pleural effusion and is thus easier to detect the tumor and to insert the needle. Although US allows for real-time surveillance of the puncture procedure, the lesion is sometimes insufficiently visualized and artifacts may hamper precise needle placement. Especially in the case of liver cirrhosis, CT imaging may sometimes be helpful in distinguishing malignant liver nodules from benign regenerative nodules^[14,15]. In addition, the CT-guided technique enables us to determine whether the tumor

nodule is completely ablated and necrotized just after the procedure by dynamic CT. We avoided any reintervention due to the remaining vital tumor tissue at the site of ablation.

The transpulmonary approach has been proven to be a safe and feasible option in the treatment of lung neoplasms although pneumothorax is a major complication^[11,12]. Pneumothorax requiring pleural drainage is the main complication of RFA for lung cancer^[11]. Lung RFA has shown itself to be a safe and feasible option for the treatment of lung cancer^[11]. In our study, the diaphragm was also punctured with RFA needle when the patient underwent CT-guided trans-pulmonary RFA for HCC. Fortunately, there was no major complication concerning diaphragm injury or massive pleural effusion. Shibata *et al*^[13] also reported that percutaneous transthoracic RFA can be used in the treatment of liver tumors in the hepatic dome. They experienced a case of moderate pleural effusion^[13], suggesting that all the patients undergoing this procedure should thus be carefully watched for pleural effusion. In our study, two of seven patients required a chest tube. Fortunately, we did not experience cases complicated by uncontrollable pneumothorax. When the patients have lung emphysema, pneumothorax may be more complicated and uncontrollable. The indication for trans-pulmonary RFA should be restricted for such cases complicated by lung emphysema.

In conclusion, CT-guided trans-pulmonary RFA for patients with HCC in the hepatic dome may be a safe and potentially effective therapy. In the cases for which artificial pleural effusion methods are not appropriate, CT guidance may therefore be a useful modality.

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RAPID COMMUNICATION

Involvement of pancreatic and bile ducts in autoimmune pancreatitis

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Abstract

AIM: To examine the involvement of the pancreatic and bile ducts in patients with autoimmune pancreatitis.

METHODS: Clinical and cholangiopancreatographic findings of 28 patients with autoimmune pancreatitis were evaluated. For the purposes of this study, the pancreatic duct system was divided into three portions: the ventral pancreatic duct; the head portion of the dorsal pancreatic duct; and the body and tail of the dorsal pancreatic duct.

RESULTS: Both the ventral and dorsal pancreatic ducts were involved in 24 patients, while in 4 patients only the dorsal pancreatic duct was involved. Marked stricture of the bile duct was detected in 20 patients and their initial symptom was obstructive jaundice. Six patients showed moderate stenosis to 30%-40% of the normal diameter, and the other two patients showed no stenosis of the bile duct. Although marked stricture of the bile duct was detected in 83% (20/24) of patients who showed narrowing of both the ventral and dorsal pancreatic ducts, it was not observed in the 4 patients who showed involvement of the dorsal pancreatic duct alone ($P=0.0034$).

CONCLUSION: Both the ventral and dorsal pancreatic and bile ducts are involved in patients with autoimmune pancreatitis.

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Key words: Autoimmune pancreatitis; Dorsal pancreatic

duct; Ventral pancreatic duct

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INTRODUCTION

Autoimmune pancreatitis is a recently described clinical entity in which autoimmune mechanisms are involved in the pathogenesis^[1-3]. It is characterized by the following clinical features: elderly male preponderance, the frequent initial symptom of jaundice, laboratory findings of serum elevation of gammaglobulin or IgG, especially IgG4, presence of autoantibodies, and a favorable response to steroid therapy. Histological findings are dense lymphoplasmacytic infiltration and fibrosis of the pancreas.

Frequently observed radiological imaging features are the enlargement of the pancreas and irregular narrowing of the main pancreatic duct. Most of the cases with autoimmune pancreatitis reveal a diffusely enlarged pancreas with a sausage-like appearance. Typical cases also show diffuse irregular narrowing of the entire main pancreatic duct. A focal type of autoimmune pancreatitis, showing focal swelling with a localized narrowing of the main pancreatic duct and upstream dilatation, is regarded as a variant of autoimmune pancreatitis^[4].

Stenosis of the bile duct frequently occurs with autoimmune pancreatitis. The stenotic portion of the bile duct is usually the lower bile duct; however, the middle and upper extrahepatic bile duct and intrahepatic bile duct are sometimes involved. There are few precise reports of cholangiopancreatographic findings in autoimmune pancreatitis^[5-7]. In the present study, we have aimed to examine the involvement of the pancreatic and bile ducts in patients with autoimmune pancreatitis.

MATERIALS AND METHODS

Patients

Between 1975 and 2003, a total of 28 patients (22 men and 6 women, average age 68.4 years) were diagnosed with autoimmune pancreatitis by the following

clinicopathological criteria: irregular narrowing of the main pancreatic duct on endoscopic retrograde pancreatography ($n=28$); pancreatic enlargement on ultrasound or computed tomography ($n=27$); hypergammaglobulinemia in excess of 2.0 g/dL ($n=15$); elevated serum IgG4 in excess of 135 mg/dL ($n=15$); presence of autoantibodies ($n=15$); characteristic histological findings in the pancreas ($n=11$); and responsiveness to steroid therapy ($n=15$).

Methods

All patients underwent endoscopic retrograde cholangiopancreatography, and 10 patients also underwent magnetic resonance cholangiopancreatography. In all the patients treated with steroids, cholangiopancreatography was performed before and after the treatment. For the purposes of this study, we divided the pancreatic duct system into three portions: the ventral pancreatic duct; the head portion of the dorsal pancreatic duct including the accessory pancreatic duct; and the body and tail of the dorsal pancreatic duct. Clinical and cholangiopancreatographic findings were evaluated.

RESULTS

Both the ventral and dorsal pancreatic ducts were involved in 24 patients, 6 of whom showed segmental narrowing of the head portion of the dorsal pancreatic duct. In four patients, only the dorsal pancreatic duct was involved, two of whom showed segmental narrowing of the body and tail of the dorsal pancreatic duct.

Stenosis of the bile duct was detected in 26 patients, including 23 patients in the lower portion of the duct, 1 patient in the middle portion, 1 patient in the lower and intrahepatic portions, and 1 patient in the middle and intrahepatic portions. A marked stricture of the bile duct was detected in 20 patients and their initial symptom was obstructive jaundice. The other 6 patients showed moderate stenosis to 30%-40% of the normal diameter.

Although marked stricture of the bile duct was detected in 83% (20/24) patients who showed narrowing of both the ventral and dorsal pancreatic ducts, it was not observed in the four patients who showed only the involvement of the dorsal pancreatic duct ($P=0.0034$).

DISCUSSION

The typical pancreatographic appearance of autoimmune pancreatitis is diffuse irregular narrowing of the main pancreatic duct involving both the ventral and dorsal pancreatic ducts^[1-3]. Some cases of autoimmune pancreatitis show segmental irregular narrowing of the main pancreatic duct^[4,7]. The segmental and diffuse types of autoimmune pancreatitis appear to represent a spectrum of the same disease rather than separate entities, as some studies^[5,8] have reported the progression of segmental narrowing to diffuse narrowing on serial endoscopic retrograde pancreatography without steroid treatment. The pancreas develops by the fusion of the dorsal and ventral pancreatic primordial buds. The main pancreatic duct is formed by the fusion of the ventral and dorsal pancreatic ducts, while the accessory pancreatic

duct is formed from the proximal portion of the dorsal pancreatic duct^[9,10]. In the present study, we have divided the pancreatic duct system embryologically into three parts: the ventral pancreatic duct, the head portion of the dorsal pancreatic duct including the accessory pancreatic duct, and the body and tail of the dorsal pancreatic duct. Both the ventral and dorsal pancreatic ducts were involved in 24 patients, while 4 patients exhibited pathologic changes only in the dorsal pancreatic duct. Although the pathogenesis of autoimmune pancreatitis is unclear, autoimmune mechanisms might have occurred only in the dorsal pancreas in these 4 patients.

Stenosis of the bile duct frequently occurs with autoimmune pancreatitis. While the stenotic portion of the bile duct is usually the lower bile duct, the upper extrahepatic and intrahepatic bile ducts are sometimes involved. In the present study, a marked stricture of the bile duct was observed in 83% (20/24) patients with narrowing of both the ventral and dorsal pancreatic ducts, while 4 patients without the involvement of the ventral pancreatic duct showed no stenosis or moderate stenosis of the bile duct and no clinical manifestation of obstructive jaundice. Diffuse infiltrations by numerous lymphocytes and plasma cells with marked fibrosis in the pancreas and bile duct characterize the histological findings of autoimmune pancreatitis^[7,11]. Periductal inflammation and marked fibrosis are the main causes of the bile duct stricture in autoimmune pancreatitis; however, fibrosis of the ventral pancreas around the lower bile duct may also be a contributing factor. In terms of the anatomical relationship between the dorsal pancreas and the common bile duct, obstructive jaundice rarely occurs without the involvement of the ventral pancreas.

In conclusion, both the ventral and dorsal pancreatic ducts are involved in a majority of the patients with autoimmune pancreatitis, while some patients show the involvement of only the dorsal pancreatic duct. Although obstructive jaundice due to stenosis of the bile duct commonly occurs with autoimmune pancreatitis, it rarely occurs in patients without the involvement of the ventral pancreas.

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Severity of ulcerative colitis is associated with a polymorphism at diamine oxidase gene but not at histamine N-methyltransferase gene

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regardless clinical evolution, and control individuals.

CONCLUSION: The His645Asp polymorphism of the histamine metabolising enzyme ABP1 is related to severity of ulcerative colitis.

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Key words: Ulcerative colitis; Pharmacogenetics; Histamine N-Methyltransferase; Diamine Oxidase

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Abstract

AIM: To analyse the role of two common polymorphisms in genes coding for histamine metabolising enzymes as it relates to the risk to develop ulcerative colitis (UC) and the clinical course of these patients.

METHODS: A cohort of 229 unrelated patients with UC recruited from a single centre and 261 healthy volunteers were analysed for the presence of Thr105Ile and His645Asp amino acid substitutions at histamine N-methyltransferase (HNMT) and diamine oxidase (ABP1) enzymes, respectively, by amplification-restriction procedures. All patients were phenotyped and followed up for at least 2 years (mean time 11 years).

RESULTS: There were no significant differences in the distribution of ABP1 alleles between ulcerative colitis patients and healthy individuals [OR (95% CI) for variant alleles = 1.22 (0.91-1.61)]. However, mutated ABP1 alleles were present with higher frequency among the 58 patients that required immunosuppressive drugs [OR (95% CI) for carriers of mutated alleles 2.41 (1.21-4.83; $P=0.006$)], with a significant gene-dose effect ($P=0.0038$). In agreement with the predominant role of ABP1 versus HNMT on local histamine metabolism in human bowel, the frequencies for carriers of HNMT genotypes or mutated alleles were similar among patients,

INTRODUCTION

Mast cells have been recognized as a key cell type involved in type I hypersensitivity. Several studies support a role of mast cells in the development of IBD. These studies indicate that among patients with IBD there is an increase in the number of mast cells and high levels of their degranulation products, such as histamine and tryptase, in the mucosa of colon and ileum.^[1,2] In addition, it has been shown that the extent of N-methylhistamine urinary excretion is nearly twice in patients with IBD than in healthy subjects, thus indicating that histamine is involved in IBD.^[3] Increased fecal levels of N-methylhistamine in patients with inflammatory bowel disease (IBD), as compared to control subjects, has also been reported.^[4,5] Further evidences support that the use of mast cell stabilizers, such as ketotifen, attenuates the severity of IBD.^[5,6]

Histamine is degraded through two enzymes, histamine N-methyltransferase (HNMT, EC. 2.1.1.8) and diamine oxidase (amiloride binding protein 1, ABP1; EC 1.4.3.6). The expression of ABP1 and HNMT in human bowel has been reported.^[7-9] The genes coding for these enzymes are polymorphic and diverse nonsynonymous single nucleotide polymorphisms (SPNs) have been described

on these genes. The *HNMT* gene, located in chromosome 1p32, shows eight SNPs, but only one of these, located in exon 4 C314T, causes amino acid substitution Thr105Ile.^[10] The variant allele is unambiguously related to decreased enzyme activity and immunoreactive protein^[11] and among Caucasian individuals corresponds to nearly 10% of alleles.^[10] Four nonsynonymous SNPs have been mapped to the *ABP1* gene, located in chromosome 7q34-36. Among these, the SNP located in exon3 C2029G codes for an altered protein with the amino acid substitution His645Asp and preliminary reports indicate that these occur with a high allele frequency, corresponding to roughly 50% alleles.^[12]

The role of histamine in IBD is sufficiently documented to allow the examination of the contribution of genetic polymorphisms in IBD. We hypothesize that individuals carrying variant alleles leading to a decreased inactivation of histamine may show altered susceptibility or a different evolution of IBD. A preliminary study involving 17 patients with ulcerative colitis (UC) and 34 patients with Crohn's disease indicated a similar frequency of *HNMT* C314T variant alleles among patients with IBD as compared to control subjects.^[13] In contrast, the same study reported an increased frequency of individuals carrying variant *ABP1* alleles among patients with IBD. However, none of the *ABP1* gene variations analyzed in such study is located within the coding region of the gene, and no evidences on a relevant effect of these mutations in *ABP1* enzyme activity or expression have been published. The present study was undertaken to investigate whether common SNPs causing amino acid substitutions on *HNMT* and *ABP1* enzymes are related to UC. For this we selected a large group of patients and controls, numerous enough to obtain conclusive evidences even if the allele frequencies differ only by 10 % among cases and controls.

MATERIALS AND METHODS

Study population

We studied a cohort of 229 Caucasian unrelated consecutive patients with ulcerative colitis recruited in the Unit of Inflammatory Bowel Disease from a single tertiary referral center (Hospital Clínico San Carlos) in Madrid, Spain. Ninety-one were women (mean age \pm SD at diagnosis 36.57 \pm 13.62), and 138 men (38.13 \pm 15.92), with a median duration of follow-up of 9 years (mean 11 years, range 2-41 years). Diagnosis of UC was based on standard clinical, radiological, endoscopic, and histologic criteria. Phenotypic details were obtained by review of clinical charts and personal interview with the patients. The same clinical questionnaire was completed for each patient. This questionnaire included: date of birth, sex, familial IBD (defined as the presence of disease in a first or second degree relative), age at diagnosis, duration of follow-up, smoking habits, history of previous surgery (tonsillectomy, appendectomy), extraintestinal clinical manifestations (articular, cutaneous, ocular, hepatic), and previous treatment as an indication of severity of disease (immunosuppressant and surgical intervention). The immunosuppressive therapy was defined by the use of azathioprine, 6-mercaptopurine and/or cyclosporine for

a minimum of 3 mo continuous treatment at standard recommended doses to control disease activity. Surgical intervention was recorded when colectomy was indicated in patients who were intractable to medical therapy and for those with massive hemorrhage, colonic perforation and unresolving toxic megacolon. Disease distribution for UC patients was simplified into two groups: extensive UC, those with total or subtotal colitis (beyond the splenic flexure), while non-extensive disease was referred to disease limited to the left colon, including patients with left side colitis, proctosigmoiditis and proctitis. Distribution was assessed macroscopically and microscopically at the time of diagnosis and at subsequent colonoscopies. The maximum extent recorded was used in the classification for this study. All patient data were recorded by a gastroenterologist from the Unit of IBD who was blind to the genotype status of each patient.

A total of 261 normal healthy unrelated controls ethnically matched with patients and from the same geographic area were recruited. All individuals were Caucasian living in Madrid and surrounding area. Control subjects were selected among the staff of the Universities and Hospitals participating in the study. One hundred and four were women (mean age \pm SD 45.42 \pm 12.91 years) and 157 men (43.51 \pm 10.62 years). Medical examination and history was obtained from every individual to exclude pre-existing disorders. Subjects with personal or familial (up to second degree relatives) antecedents of IBD, autoimmune or allergic diseases were excluded. The protocol was approved by the Ethics Committee of the Hospital Clínico San Carlos (Madrid), and all patients and controls were included in the study after informed consent. All UC patients and over 95% of the healthy subjects requested agreed to participate in the present study.

Genotyping

Genomic DNA was obtained from peripheral leukocytes and purified according to standard procedures. The presence of the SPNs was investigated by amplification-restriction and electrophoresis in agarose gels. The analysis of the *HNMT* C314T SNP was carried out after the amplification of a gene fragment comprising exon 4 using the following primers (based on the published human *HNMT* sequence Gene Bank Accession No. U44109): GAA AAA CGT TCT TTC TAT CTG TTT GTA TAT AA and ATT TGG GCA GAT CAT GGT CAC TTG T. After an initial step of 2 min at 94 °C, PCR amplification was carried out for 40 cycles of 25 s at 94 °C, 1 min at 52 °C, and 1 min at 72 °C, and a final extension period of 5 min at 72 °C. The 394 base pairs (bp) PCR product was incubated with the endonuclease *EcoRV* that digests the mutated allele in two fragments of 179 and 215 base pairs, leaving intact the wild-type sequence.

Regarding the C2029G SNP within the *ABP1* gene, the following primers (based on the diamine oxidase gene sequence Gene Bank Accession No. X78212) were used: GGT CAC CTG AAC CCG GTT AAC and TTG TGA CCT CTG AAC TTG CCG. After an initial step of 2 min at 94 °C, PCR amplification was carried out for 40 cycles of 25 s at 94 °C, 1 min at 61 °C, and 1 min at 72 °C, and a final extension period of 5 min at 72 °C. The amplified

Table 1 *HNMT* genotypes and allele frequencies among overall UC patients and healthy controls

| Genotype | UC patients | | Healthy controls | | OR (95% CI) | <i>p</i> |
|----------------|-------------|------------------|------------------|------------------|------------------|----------|
| | <i>n</i> | % (95% CI) | <i>n</i> | % (95% CI) | | |
| C/C | 161 | 82.6 (77.2-87.9) | 193 | 78.8 (73.7-83.9) | 1.27 (0.79-2.05) | 0.33 |
| C/T | 32 | 16.4 (11.2-21.6) | 48 | 19.6 (14.6-24.6) | 0.75 (0.45-1.23) | 0.34 |
| T/T | 2 | 1.0 (0-2.4) | 4 | 1.6 (0-3.2) | 0.60 (0.13-2.85) | 0.50 |
| Total subjects | 195 | | 245 | | | |
| C allele | 354 | 90.8 (87.9-93.6) | 434 | 88.6 (85.8-91.4) | 1.27 (0.80-2.02) | 0.29 |
| T allele | 36 | 9.2 (6.4-12.1) | 56 | 11.4 (8.6-14.2) | | |
| Total alleles | 390 | | 490 | | | |

Table 2 *ABP1* genotypes and allele frequencies among overall UC patients and healthy controls

| Genotype | UC patients | | Healthy controls | | OR (95% CI) | <i>p</i> |
|----------------|-------------|------------------|------------------|------------------|------------------|----------|
| | <i>n</i> | % (95% CI) | <i>n</i> | % (95% CI) | | |
| C/C | 101 | 44.1 (37.7-50.5) | 137 | 52.5 (46.4-58.5) | 0.71 (0.49-1.04) | 0.064 |
| C/G | 115 | 50.2 (43.7-56.7) | 108 | 41.4 (35.4-47.3) | 1.43 (0.98-2.08) | 0.050 |
| G/G | 13 | 5.7 (2.7-8.7) | 16 | 6.1 (3.2-9.0) | 0.92 (0.41-2.08) | 0.832 |
| Total subjects | 229 | | 261 | | | |
| C allele | 317 | 69.2 (65.0-73.4) | 382 | 73.2 (69.4-77.0) | 1.21 (0.92-1.60) | 0.17 |
| G allele | 141 | 30.8 (26.6-35.0) | 140 | 26.8 (23.0-30.6) | | |
| Total alleles | 458 | | 522 | | | |

476 bp fragment contains a constitutive restriction site for the endonuclease *AnaII*, and a variant-allele specific restriction site. After endonuclease digestion the wild type gene yields fragments of 369 and 107 bp whereas the mutated gene is digested to fragments of 252, 117 and 107 bp.

Statistical analysis

The frequencies for the *HNMT* and *ABP1* SNPs were estimated by counting genes and calculating sample proportions. Case-control analyses were performed with the χ^2 statistics or Fisher exact test, each when adequate. The association between *HNMT* and *ABP1* mutations and phenotypic characteristics of UC was estimated by the odds ratio (OR) with the 95% confidence interval (CI). To assess whether *HNMT* and *ABP1* variants influence synergically upon the course of UC, subjects were classified as carriers and noncarriers of nonsynonymous SNPs. The above cited test was used for the comparison of carriers and noncarriers. Logistic regression analysis was performed to assess whether *HNMT* and *ABP1* mutations were correlated with a particular clinical phenotype. Association was expressed as OR with 95% CI. A two-tailed *P* value equal or less than 0.05 was considered as significant. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 10.07 for Windows (SPSS Inc., Chicago, Ill. USA).

RESULTS

The frequency analyses for *HNMT* and *ABP1* polymorphisms are summarized in Tables 1 and 2. The *HNMT* genotype (Table 1) was analyzed in 195 patients and no differences with controls in genotype distribution were observed. The mutated *HNMT* allele frequencies were also similar among patients and controls, 9.2 and 11.4%, respectively. The OR (95% CI) value for the mutated allele was 0.79 (0.51-1.22).

In contrast, Table 2 shows that the distribution of *ABP1* genotypes differs between UC patients and healthy controls, and that these differences are in the limit of statistical significance for individuals heterozygous for variant alleles (OR = 1.43; 95% CI = 0.98-2.08; *P* = 0.050). The mutated *ABP1* allele frequencies are increased at a non-significant level among patients (OR = 1.21; 95% CI = 0.92-1.60). Regarding the interaction of both

genotypes, the frequencies for individuals with both mutated genotypes were similar among UC patients (12.2%) and healthy controls (10.8%). Among UC individuals with mutated *HNMT* genotypes, the frequencies for C/C, C/G and G/G *ABP1* genotypes were 38.2%, 55.9% and 5.9%, respectively. Inversely, among UC individuals with mutated *ABP1* genotypes, the frequencies for the *HNMT* genotypes C/C C/T and T/T were 80.6%, 18.4% and 1.0%, respectively. These allele frequencies are similar to those obtained in overall patients, thus indicating that no *HNMT/ABP1* gene interaction is observed among UC patients.

In order to obtain further information on the effect of both studied polymorphisms on phenotypic and clinical characteristics of patients, these were grouped as carriers and non-carriers of the corresponding mutations, either in heterozygosity or homozygosity. *ABP1* genotypes are similarly distributed when patients are subdivided according to gender, mean age at diagnosis, mean duration of the disease, family history of IBD, smoking habits, previous appendectomy or tonsillectomy, extent of UC, and extraintestinal clinical manifestations, including cutaneous, articular, ocular and hepatic manifestations of the disease (Table 3).

However, *ABP1* genotypes were linked to parameters closely related to the severity of the disease, i.e. need of immunosuppressant therapy and/or need for surgery. Among the 58 patients that required immunosuppressive drugs, 18 of them ultimately needing colectomy due to unresponsiveness of the disease, 41 (70%) were carriers of *ABP1* mutations, 6 of them in homozygosity, the proportion of carriers of the mutated allele being significantly higher than that found in the remaining patients [OR (95% CI) = 2.41 (1.21-4.83), *P* < 0.006] (Table 3). Moreover, the probability to need immunosuppressant therapy was higher in homo- (OR = 4.24) than in heterozygote (OR = 2.16) carriers of *ABP1* mutated alleles (Chi-square for linear trend 8.36, *P* = 0.0038).

The other clinical incidence related to severity of UC we have analyzed is the need for surgical intervention. Nineteen out of the 23 patients requiring intervention were also carriers of mutations at the *ABP1* gene (OR = 2.50; 95% CI = 1.28-4.91; *P* = 0.003). It should be noted, however, that 18 of these individuals are included in the group of patients needing immunosuppressant therapy.

Table 3 Phenotypic characteristics among UC patients classified as carriers or non-carriers of mutations at the *ABP1* gene

| Phenotypic characteristics | Carriers <i>n</i> = 128 | Non-carriers <i>n</i> = 101 | OR (95%CI) <i>P</i> value |
|---|----------------------------|--------------------------------|------------------------------|
| Male:Female | 76:52 | 62:39 | 0.79 (0.52-1.62) 0.86 |
| Mean age at diagnosis, yr (range) | 37.9 (6-83) | 37.1 (17-81) | <i>P</i> =0.64 |
| Mean duration of disease, yr (range) | 11.5 (2-39) | 11.0 (2-41) | <i>P</i> =0.58 |
| Family history of IBD, <i>n</i> (%) | 31 (24.2) | 21 (20.8) | 1.22 (0.62-2.39) 0.54 |
| Smokers at diagnosis, <i>n</i> (%) | 28 (21.9) | 22 (21.8) | 1.01 (0.51-1.98) 0.99 |
| Previous appendectomy, <i>n</i> (%) | 5 (3.9) | 6 (6.9) | 0.64 (0.16-2.47) 0.54 |
| Previous tonsillectomy, <i>n</i> (%) | 11 (8.6) | 14 (13.9) | 0.58 (0.23-1.55) 0.29 |
| Extent of UC | | | |
| Extensive, <i>n</i> (%) | 53 (41.4) | 42 (41.6) | 1.33 (0.77-2.30) 1 |
| Non-extensive, <i>n</i> (%) | 75 (58.6) | 59 (58.4) | |
| Extraintestinal clinical manifestations | | | |
| Cutaneous, <i>n</i> (%) | 23 (17.9) | 10 (9.9) | 1.99 (0.85-4.76) 0.09 |
| Articular, <i>n</i> (%) | 49 (38.3) | 28 (27.7) | 1.64 (0.90-2.99) 0.09 |
| Ocular, <i>n</i> (%) | 3 (2.3) | 4 (3.9) | 0.58 (0.10-3.17) 0.7 |
| Hepatic, <i>n</i> (%) | 31 (24.2) | 14 (13.9) | 1.99 (0.94-4.22) 0.07 |
| Therapy categories | | | |
| Immunosuppressants, <i>n</i> (%) | 41 (70) | 17 (30) | 2.41 (1.21-4.83) 0.006 |
| Surgery, <i>n</i> (%) ¹ | 19 (83) | 4 (17) | 2.50 (1.28-4.91) 0.003 |

¹Eighteen of these patients are also included in the subgroup of patients receiving immunosuppressive therapy. Four of the remaining 5 patients in the surgery group were carriers of the variant *ABP1* allele.

In the remaining 5 patients colectomy was indicated by acute complications of the disease, as colonic perforation or toxic megacolon.

HNMT genotypes are similarly distributed when patients are subdivided according gender, mean age at diagnosis, mean duration of the disease, family history of IBD, smoking habits, previous appendectomy or tonsillectomy, extent of UC, and extraintestinal clinical manifestations, including cutaneous, articular, ocular and hepatic manifestations of the disease (Table 4). Moreover, no statistically significant differences were observed regarding *HNMT* allele frequencies among patients requiring either immunosuppressive drugs (OR = 1.03; 95 % CI = 0.44-2.42) or surgical intervention (1.94; 0.72 - 5.24), respectively. Gene interactions for these subgroups of patients were calculated as described above and no differences were found in relation to the remaining patients.

Table 4 Phenotypic among UC patients classified as carriers or non-carriers of mutations at the *HNMT* gene

| Phenotypic characteristics | Carriers <i>n</i> = 34 | Non-carriers <i>n</i> = 161 | OR (95% CI) <i>P</i> value |
|--------------------------------------|---------------------------|--------------------------------|--|
| Male:Female | 20:14 | 91:70 | 1.10 (0.49-2.49) 0.81 |
| Mean age at diagnosis, yr (range) | 35 (6-83) | 38 (17-81) | <i>P</i> =0.24 |
| Mean duration of disease, yr (range) | 12.4 (2-39) | 11.3 (2-41) | <i>P</i> =0.11 |
| Family history of IBD, <i>n</i> (%) | 8 (23.5%) | 35 (21.7%) | 1.11 (0.42-2.85) 0.82 |
| Smokers at diagnosis, <i>n</i> (%) | 8 (23.5%) | 31 (19.3%) | 1.29 (0.48-3.36) 0.57 |
| Previous appendectomy, <i>n</i> (%) | 2 (5.9%) | 6 (3.7%) | 1.61 (0.21-9.47) 0.63 |
| Previous tonsillectomy, <i>n</i> (%) | 2 (5.9%) | 17 (10.5%) | 0.53 (0.08-2.57) 0.54 |
| Extent of UC | | | |
| Extensive, <i>n</i> (%) | 15 (44.1%) | 64 (39.8%) | 1.20 (0.53-2.69) 0.63 |
| Non-extensive, <i>n</i> (%) | 19 (55.9%) | 97 (60.2%) | |
| Extraintestinal manifestations | | | |
| Cutaneous, <i>n</i> (%) | 6 (17.6%) | 19 (11.8%) | 1.60 (0.52-4.66) 0.4 |
| Articular, <i>n</i> (%) | 15 (44.1%) | 47 (29.2%) | 1.91 (0.84-4.35) 0.09 |
| Ocular, <i>n</i> (%) | 1 (2.9%) | 5 (3.1%) | 0.95 (0.02-8.86) 1 |
| Hepatic, <i>n</i> (%) | 5 (14.7%) | 27 (16.8%) | 0.86 (0.26-2.60) 0.77 |
| Therapy categories | | | |
| Immunosuppressants, <i>n</i> (%) | 8 (23.5%) | 37 (23%) | 1.03 ¹ (0.39-2.65) <i>P</i> =0.94 |
| Surgery, <i>n</i> (%) | 6 (17.6%) | 16 (9.94%) | 1.94 ¹ (0.62-5.91) 0.20 |

¹As compared with the remaining patients.

DISCUSSION

Nonrandom clustering of distinct diseases related to immunity supports that a common set of susceptibility genes may be involved in the etiology of immune diseases. A total of 18 clustered autoimmune loci have been reported, and among them, loci located at chromosomes 1, 3, 7, 12 and 16 have been reported to be linked to IBD.^[14] Among these, chromosome 7 accumulates the highest density of autoimmune loci in man. Five loci significantly related to UC were identified in chromosome 7, where *ABP1* gene is located, with *P* values ranging from 0.003 to 0.00001.^[14] Diverse gene polymorphisms have been reported to be linked to UC extension or severity, including *MDR1*, *HLA*, or *MUC3*.^[15-16]

It has been shown that *ABP1* enzyme activity is decreased in patients with IBD, but the reason for such decrease remains unknown. Such alteration may be explained in part by polymorphisms on the *ABP1* gene that have been recently described. This is the first study analysing SNPs causing amino acid substitutions at *ABP1*

enzyme in patients with UC and, with the exception of a preliminary study carried out in a small group of patients,^[13] this is also the first one that studies *HNMT* polymorphism in UC patients. It should be noted that the *HNMT* allele frequencies observed in this study are consistent with these described previously,^[10,11] and that the frequency for the His645Asp *ABP1* polymorphism is only based on preliminary reports obtained in a limited number of individuals. In this study we have analysed a total of 456 patients and controls (912 alleles) and we have shown that in the analysed Caucasian population the mutated allele frequency observed was lower than initially expected, ranging from 25% to 30% (Table 2).

In this study we have shown that the severity of UC is influenced by a nonsynonymous *ABP1* polymorphism. Previous findings indicate that *ABP1* enzyme activity in bowel mucosa was about 50% decreased among patients with IBD, as compared to healthy individuals,^[7,17,18] thus supporting the hypothesis that individuals carrying mutations at *ABP1* gene may show altered susceptibility to UC and greater activity of the disease. The lack of association of *HNMT* polymorphisms with UC may be related to the relative contribution of *HNMT* to histamine metabolism in bowel tissue, since *HNMT* activity is almost negligible in bowel tissue as compared to *ABP1* activity. It has been reported a mean *HNMT* enzyme activity in human intestine of 0.47 nmol (min/mg),^[9] whereas *ABP1* enzyme activity in human intestine is nearly 150 nmol (min/mg).^[18]

Histamine release into the bowel is increased in IBD patients compared with controls, and secretion of histamine is related to the disease activity. Markedly elevated mucosal histamine levels were observed in patients with UC,^[1] and increased levels of N-methylhistamine, a stable metabolite of histamine, were detected in the urine of patients with UC^[3]. In addition, the production of histamine and the urinary excretion of N-methylhistamine are coupled with the degree of inflammation in CD and with the extension of the mucosal surface involved in UC. N-methylhistamine urinary excretion rate was also related to the severity of mucosal inflammation, as it is higher in patients with moderate and high inflammatory mucosa than in patients with only mildly or non-inflammatory mucosa.^[3] On the contrary, UC is confined to the mucosa of the colon. Extracellular histamine is metabolized to imidazole acetic acid primarily by *ABP1* at the gut mucosal surface. Different polymorphisms at the *ABP1* gene might affect in different degrees the catabolic rate of histamine in the gut mucosal surface; hence, we can speculate that the association we have found between the carrier state of the His645Asp substitution and the more frequent need to use immunosuppressant therapy or surgery to control the disease depends on a slower rate of histamine metabolic inactivation. Therefore, this specific mutation could be a marker of refractivity to the standard therapies for UC (sulfasalazine, aminosalicylates and glucocorticoids).

Many symptoms of patients with IBD may be explained taking in consideration the known effects of histamine. Histamine is a key mediator responsible for diarrhea, contraction of smooth muscle, modulation of immune response, or mediation of pain in UC. Higher disease severity may result from a reduction of *ABP1* activity in

UC. In this subgroup of patients with a genetically reduced rate of *ABP1* activity, the therapeutic use of inhibitors of mast cell degranulation could result particularly useful. This family of drugs has been evaluated, with conflicting results, in patients with IBD, but without considering the genetic-induced differences in histamine metabolism.^[5] The association between UC and allergic diseases could not be evaluated in the present study because the absence of personal or familial antecedents of allergy was a prerequisite to include subjects in the control group. Among UC patients, no significant differences in the frequency of allergy were identified when the *HNMT* of *ABP1* polymorphisms were considered.

The results obtained in this study provide an additional support for a key role of histamine in UC. However, it is to be noted that the *ABP1* gene is adjacent to *NOS3* gene for which there are variant alleles that seem to have differential effects on the inflammatory process.^[19] Thus it cannot be ruled out that linkage disequilibrium between *ABP1* and *NOS3* variant alleles may be related to the findings obtained in the present study. In addition, it should be emphasized that polymorphisms on genes coding for histamine-metabolising enzymes may not be the only cause for increased histamine levels in patients with UC. In fact, it has been shown that intestinal mast cell secretion and release of histamine is increased in patients with IBD,^[20,21] and this may be related to variations in histamine synthesis. Presently no variations affecting the histidine decarboxylase gene (*HDC*) have been reported. Once the analysis of such gene variations is completed, further studies should explore the role of variability in the *HDC* gene in IBD.

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Serum interleukin-1 receptor antagonist is an early indicator of colitis onset in $G\alpha i2$ -deficient mice

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INTRODUCTION

Inflammatory bowel disease (IBD), or ulcerative colitis (UC) and Crohn's disease (CD), is characterized by inflammation in the intestinal tract without any evident infection. The pathogenetic mechanisms underlying the disease induction and progression is still unclear, although clinical studies and animal models have offered insights into the pathogenesis of IBD^[1]. It is also of prognostic value in finding analyses enabling early detection of relapses, thus being able to define medical treatment and possibly cease the disease progression at an early stage. Alternatively, if the patients are at a higher risk of relapse, a more intense clinical contact is necessary^[2]. Exploring the pathogenesis of IBD and finding early markers of the disease activity are two approaches that might benefit each other. In this context, on the experimental basis, it is of great value to continuously follow animals that are born healthy, therefore displaying a disease-free interval, but pre-disposed for IBD. G protein $\alpha i2$ deficient mice develop a chronic intestinal inflammation much resembling UC in human beings, including the development of adenocarcinomas^[3]. Since the mice are born healthy and develop disease at the age of 12-25 wk, it is possible to study the pathogenesis as well as the effect of interventions initiated before the induction of the disease or during ongoing inflammation. Furthermore, the murine model of $G\alpha i2$ -deficient mice enables studies on early markers of IBD induction/progression.

The importance of the commensal flora for the induction of experimental IBD is reported in several different models where animals under specific pathogen-free conditions develop intestinal inflammation, while they remain healthy or develop only mild inflammation in a germ-free environment^[4-7]. This is true also for $G\alpha i2$ -deficient mice (Birnbaumer, personal communication). As far as we know, the toll-like receptors (TLRs) form one of the primary sensors of the immune system in detecting bacterial structures. Interestingly, several components of the TLR intracellular (IC) signaling pathway are shared also by interleukin-1 receptor type I (IL-1RI) and the

Abstract

AIM: To study the serum concentration of IL-1 β , IL-1 receptor antagonist (IL-1Ra) and IL-18 in $G\alpha i2$ -deficient mice at the age of 6 (healthy), 12 (pre-colitic) and 24 wk (colitic) and in healthy control mice.

METHODS: At the time of killing, serum samples were collected and IL-1 β , IL-1Ra and IL-18 levels were measured using enzyme-linked immunosorbent assays.

RESULTS: Serum concentration of IL-1Ra was significantly increased in pre-colitic (median: 524 ng/L; $P=0.02$) and colitic (450 ng/L; $P=0.01$), but not in healthy (196 ng/L) $G\alpha i2$ -deficient mice as compared with controls (217 ng/L). Serum concentrations of IL-1 β did not differ between $G\alpha i2$ -deficient mice and their controls, irrespective of age, IL-18 was significantly increased in colitic, but not in pre-colitic mice compared with controls (510 ng/L vs 190 ng/L; $P=0.05$).

CONCLUSION: The increased serum concentrations of IL-18 and IL-1Ra in established diseases are suggested as markers of ongoing colitis. Interestingly, the significantly increased serum concentration of IL-1Ra in pre-colitic mice is found to be an early marker of disease progression.

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Key words: IL-1Ra; IL-18; IBD; Colitis; Mice

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interleukin-18-receptor (IL-18R). Since these IC signaling mediators, e.g. IL-1 receptor-associated kinase-1 (IRAK-1)^[8] and -M (IRAK-M)^[9], are highly involved in the regulation of TLR signaling, one might expect an early and important role of IL-1RI and IL-18R in regulating inflammation induced via TLR. IL-18 is a co-stimulator for IFN- γ production^[10] and the *G α i2*-deficient mice is known to display an immunological dysregulation characterized by high IFN- γ production in intestinal compartments^[11]. The main sources of IL-18 in the intestinal tract are intestinal epithelial cells (IEC), macrophages and dendritic cells (DCs)^[12-14]. The importance of IL-18 for the induction of colitis has previously been shown in the model of 2,4,6-trinitrobenzene sulfonic acid (TNBS) induced colitis in which IL-18-deficient mice do not develop disease, whereas a Crohn's-like disease develops in the wild-type mice^[15]. The interleukin-1-receptor antagonist (IL-1Ra) binds to the IL-1RI and blocks the pro-inflammatory function of IL-1 in colitis^[16] and neutralization of IL-1Ra results in the exacerbation of colitis^[17]. Though the IL-1Ra production is significantly increased by IEC during inflammation in CD and UC patients^[18], an increased IL-1/IL-1Ra ratio in the intestinal mucosa is still seen in these patients^[19]. The spontaneous production of IL-1 β is highly increased (30-fold) in colons of *G α i2*-deficient mice with colitis as compared with wild-type mice, while the increase in TNF production is more modest (2-fold)^[11]. The aim of this study was to analyze the serum concentrations of IL-1 β , IL-1Ra, and IL-18 in pre-colitic and colitic *G α i2*-deficient mice in order to find early serum markers of colitis.

MATERIALS AND METHODS

Mice

Mice were kept and bred at the animal facility of the Department of Experimental Biomedicine, Göteborg University, Sweden. They were kept under standard conditions of temperature and light, and fed with standard laboratory chow and water *ad libitum*. The procedure of *G α i2* gene disruption has been described in detail elsewhere^[5]. *G α i2*-deficient mice on a mixed C57BL/6X129SvEv background were backcrossed four generations into 129SvEv and then intercrossed. Homozygous *G α i2*^{-/-} males were bred with heterozygous females and the offspring were genotyped by polymerase chain reaction (PCR) analysis. *G α i2*-deficient mice (*G α i2*^{-/-}) on this background developed colitis between 12 and 25 wk of age, irrespective of sex, while the *G α i2*^{+/-} similar to *G α i2*^{+/+} mice stayed healthy. The mice were killed at the age of 6 (healthy), 12 (pre-colitic), and 24 (colitic) wk. At the time of killing, a gross examination of the intestines was performed to look for the presence of colitis. Colitis was scored: 0 = normal; 1 = mild colitis; 2 = moderate colitis and 3 = severe colitis. Blood samples were taken and spleens were weighed. Both male and female mice were included in the study. Heterozygous mice were used as controls throughout the study. The experiments were approved by the Göteborg Animal Ethics Committee.

Cytokine analyses

Serum concentrations of IL-1 β and IL-1Ra were estimated using Quantikine mouse immunoassays (R&D Systems, Minneapolis, MN, USA) and IL-18 was measured using a commercially available enzyme immunoassay (MBL, Nagoya, Japan). Detection limits were: IL-1 β : 3 ng/L, IL-1Ra: 7 ng/L and IL-18: 25 ng/L. All the assays were performed in accordance with the manufacturer's instructions.

Statistical analysis

Data were analyzed using the non-parametric Mann-Whitney *U* test. A value of $P \leq 0.05$ was considered as statistically significant.

RESULTS

Serum IL-18 but not IL-1 β is a marker of ongoing colitis

We examined the serum concentrations of IL-18 and IL-1 β in the colitic animals as well as their littermate controls. As expected heterozygous controls displayed no or very low serum IL-1 β concentration, but also the 24-wk-old *G α i2*-deficient mice had serum levels of IL-1 β as compared to *G α i2*^{+/-} controls (median: 18 ng/L *vs* 9 ng/L; ns, not shown). The levels of circulating IL-18 in heterozygous controls were clearly above the detection limit of the assay. However, in contrast to the IL-1 β serum levels, the IL-18 concentration was significantly increased in the colitic mice as compared with control animals (Figure 1A). One of the *G α i2*^{+/-} mice displayed a highly increased IL-18 level as compared with the controls. This mouse appeared healthy and was included in the statistical analysis.

Serum IL-1-receptor antagonist is an early indicator of colitis onset

The serum concentrations of IL-1Ra were analyzed in *G α i2*-deficient mice and their littermate controls. In parallel with IL-18, serum IL-1Ra was also easily detected in control animals. In contrast to IL-1 β that was not increased in colitic animals, the IL-1Ra concentrations were significantly higher than the controls (Figure 1B). Interestingly, similar to the colitic animals, pre-colitic *G α i2*^{-/-} mice also had significantly higher serum IL-1Ra levels than their controls. In contrast, serum concentrations of IL-18 and IL-1 β were not raised in the 12-wk-old pre-colitic animals as compared with their controls (median 325 ng/L *vs* 190 ng/L; and 10 ng/L *vs* 9 ng/L).

In the 12-wk-old group of pre-colitic *G α i2*^{-/-} mice, 6 mice did not show any signs of colitis at gross examination, two had mild disease, one moderate and one was judged to have severe colitis. In the 24-wk-old group only one mouse had a normal colon by gross examination, two had mild disease, three moderate and three had severe disease.

The IL-18 concentrations were found higher in colitic than in pre-colitic mice, which was in parallel with a disease progression between 12 and 24 wk of age. However, this was not seen with regard to the serum IL-1Ra concentration. In our previous study *G α i2*-deficient cells showed exaggerated cytokine production upon

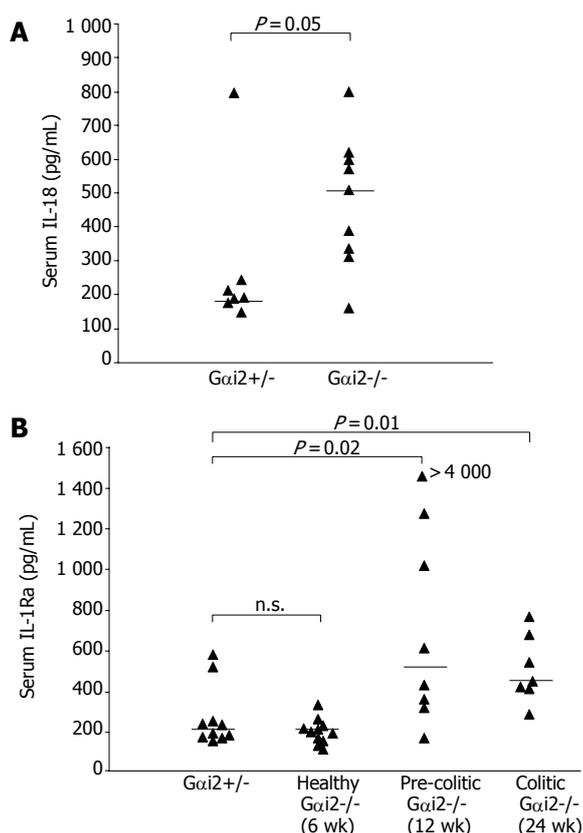


Figure 1 A: Serum IL-18 concentrations in 24-wk-old *Gαi2*-deficient mice ($n=9$) and their heterozygous littermates (controls; $n=7$). Data were analyzed using the non-parametric Mann-Whitney U test. A value of $P \leq 0.05$ was regarded as statistically significant. The horizontal bars mark the median values within each group; **B:** Serum IL-1Ra concentrations in 6-wk-old (healthy; $n=11$), 12-wk-old (pre-colitic; $n=8$), and 24-wk-old (colitic; $n=7$) *Gαi2*-deficient mice and their heterozygous controls ($n=10$). Data were analyzed using the non-parametric Mann-Whitney U test. A value of $P \leq 0.05$ was regarded as statistically significant. The horizontal bars mark the median values within each group.

stimulation, even if they were derived from a non-colitic genetic background^[20]. To examine whether the IL-1Ra concentration was increased upon colitis onset, we also analyzed the serum IL-1Ra concentration in 6-week-old (healthy) *Gαi2*-deficient mice. The 6-wk-old *Gαi2*^{-/-} mice had similar serum level of IL-1Ra to the *Gαi2*^{+/-} control animals. Thus, serum IL-1Ra concentrations increased upon colitis onset which is not merely a consequence of the *Gαi2* deficiency (Figure 1B).

Spleen weight seemed to be also correlated with the stage of the disease. The median weight of 132 mg in 12-wk-old mice, was significantly higher than in *Gαi2*^{+/-} mice (84 mg; $P=0.03$). The 24-wk-old mice displayed a higher spleen weight (222 mg) than not only the controls, but also the 12-wk-old mice ($P=0.03$). Interestingly, the two 12-wk-old mice with severe and moderate colitis judged by gross examination demonstrated the highest spleen weight and the highest serum IL-1Ra concentrations: 271 mg and >4000 ng/L in mouse with severe colitis and 180 mg and 1276 ng/L in mouse with moderate colitis.

DISCUSSION

We analyzed serum concentrations of IL-18, IL-1 β and the IL-1 receptor antagonist in *Gαi2*-deficient mice on

a colitis-prone genetic background. Since mice are born healthy and develop colitis at the age of approximately 12-25 wk, we were able to examine the mice at a “pre-colitic” (12 wk of age) and a “colitic” age (24 wk). Our results showed that the serum IL-18 concentration was significantly increased in *Gαi2*-deficient mice at 24 wk of age, a time when most of the mice displayed moderate or severe colitis, as compared with the controls. At this time-point serum IL-1Ra concentrations were also significantly increased, whereas the serum IL-1 β levels were similar in *Gαi2*-deficient and control mice. The 12-wk-old, “pre-colitic” *Gαi2*-deficient mice did not display increased serum levels of neither IL-18 nor IL-1 β . In contrast, the serum concentration of IL-1Ra was significantly increased in these mice. Our results suggest a potential clinical importance of measuring serum concentrations of IL-18 and IL-1Ra in IBD patients to determine the status of the inflammatory disease. The value of measuring serum IL-18 might be related to confirming ongoing colitis, additional information could be obtained by examining the serum IL-1Ra concentration. In fact, serum concentration of IL-1Ra is an early indicator for the onset of colitis.

We know from previous studies on *Gαi2*-deficient mice that the production of IL-1 β is highly increased locally in the large intestine during ongoing colitis^[11]. However, the present study clearly shows that such a dysregulation of the IL-1 β production is not reflected in the systemic circulation. In contrast to IL-1 β , IL-18 is easily detected in the blood in healthy individuals, of both mouse and man, which enables evaluation of fluctuations in IL-18 concentrations during the disease. The finding of significantly increased IL-18 levels in the 24-wk-old colitic mice is not very surprising, inasmuch as the intestinal inflammation of the *Gαi2*-deficient mice is characterized by high IFN- γ production, with IL-18 being a co-stimulator for IFN- γ production. It has been previously shown that serum IL-18 was higher in patients with CD than healthy blood donors^[21]. A recent report also showed increased plasma IL-18 level in patients with moderate or severe UC, but not in patients with mild disease^[22]. Our results confirm a role of IL-18 levels measurement in established colitis. However, it seems that IL-18 is not as sensitive as IL-1Ra in indicating onset of colitis, or perhaps not as useful as an early indicator of a relapse as compared with IL-1Ra. We found that although there were clearly detectable levels of IL-1Ra in young healthy *Gαi2*-deficient mice, the levels were similar to what was found in the control mice. In contrast, the 12-wk-old “pre-colitic” *Gαi2*-deficient mice displayed significantly higher serum IL-1Ra concentrations than their controls. In the healthy mucosa, IEC is capable of producing IL-1Ra^[18] which might be one of the sources for the serum IL-1Ra detected in healthy *Gαi2*^{-/-} mice and their *Gαi2*^{+/-} littermates. During intestinal inflammation, IEC increased the production of IL-1Ra, but a decreased IL-1Ra:IL-1 ratio was nevertheless seen^[23]. One reason for finding IL-1Ra but not IL-1 β in the circulation at the onset and in established colitis might simply be that the receptor antagonist is produced excessively as compared with the cytokine and is therefore more easily detected. Another explanation could of course be that the IL-1Ra is systemically derived. Although the visceral

organs of $\text{G}\alpha\text{i}2$ -deficient mice were previously screened microscopically without finding any abnormalities, except the colon^[3], splenomegaly seems to be correlated with disease progression in $\text{G}\alpha\text{i}2$ -deficient mice. One possible tissue origin of serum IL-1Ra in healthy individuals, except IEC, is the adipose tissue^[24,25]. An increased serum IL-1Ra concentration in the older $\text{G}\alpha\text{i}2$ -deficient can hardly be related to increased BMI. In contrast from previous results we found that serum leptin, also originating from white adipose tissue, is decreased upon colitis onset in $\text{G}\alpha\text{i}2$ -deficient mice^[26]. Furthermore, the 12-wk-old mouse displaying severe colitis had a substantial weight reduction as compared with the other mice in the group and still highly increased serum IL-1Ra concentration.

In conclusion, our results show that while circulating IL-18 and IL-1Ra are markers of established intestinal inflammation, IL-1Ra is also an early indicator of onset of colitis in $\text{G}\alpha\text{i}2$ -deficient mice. We therefore suggest that IL-1Ra might be a useful serological marker of disease progression in IBD at an early stage as well as at a relapse.

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Genetic alterations in benign lesions: Chronic gastritis and gastric ulcer

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nign lesions evidences chromosomal instability in early stages of gastric carcinogenesis associated with *H pylori* infection, which may confer proliferative advantage. The increase of p53 protein expression in CG and GU may be due to overproduction of the wild-type protein related to an inflammatory response in mucosa.

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Key words: Aneuploidies; *TP53* gene; p53 protein; Gastritis; Gastric ulcer

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Abstract

AIM: To investigate the occurrence of chromosome 3, 7, 8, 9, and 17 aneuploidies, *TP53* gene deletion and p53 protein expression in chronic gastritis, atrophic gastritis and gastric ulcer, and their association with *H pylori* infection.

METHODS: Gastric biopsies from normal mucosa (NM, $n = 10$), chronic gastritis (CG, $n = 38$), atrophic gastritis (CAG, $n = 13$) and gastric ulcer (GU, $n = 21$) were studied using fluorescence *in situ* hybridization (FISH) and immunohistochemical assay. A modified Giemsa staining technique and PCR were used to detect *H pylori*. An association of the gastric pathologies and aneuploidies with *H pylori* infection was assessed.

RESULTS: Aneuploidies were increasingly found from CG (21%) to CAG (31%) and to GU (62%), involving mainly monosomy and trisomy 7, trisomies 7 and 8, and trisomies 7, 8 and 17, respectively. A significant association was found between *H pylori* infection and aneuploidies in CAG ($P = 0.0143$) and GU ($P = 0.0498$). No *TP53* deletion was found in these gastric lesions, but p53-positive immunoreactivity was detected in 45% (5/11) and 12% (2/17) of CG and GU cases, respectively. However, there was no significant association between p53 expression and *H pylori* infection.

CONCLUSION: The occurrence of aneuploidies in be-

INTRODUCTION

Gastric carcinogenesis is considered a multi-step and multi-factorial process that proceeds from normal gastric mucosa with epithelial hyperproliferation to chronic gastritis with variable degrees of atrophy, intestinal metaplasia, dysplasia, and ultimately carcinoma^[1]. In most cases, chronic gastritis, frequently associated with *H pylori* infection, precedes the formation of gastric cancer, and a great proportion of the clinical tumors occurs in connection with severe atrophic gastritis and extensive intestinal metaplasia^[2]. Efforts have been made to identify changes of a precancerous nature in the gastric epithelium. The identification of such changes may help to prevent the occurrence of gastric cancer^[3].

Gastritis may also play a role in the pathogenesis of peptic ulcer disease by impairing the resistance of the gastric mucosa and consequently increasing the risk of ulcer^[3]. In practice, the cancer risk in peptic ulcer disease applies exclusively to gastric ulcer. Epidemiological data on the incidence of peptic ulcer disease with gastric cancer suggest that ulcer disease may not be an important precursor of gastric cancer. However, possible links between these two are suggested by a similar relationship with chronic gastritis^[2].

H pylori infection is accepted as the major causal factor for the histological features leading to severe gastroduodenal disease, including chronic gastritis, peptic

ulcer disease, intestinal-type gastric cancer and gastric MALT lymphoma. Only a small proportion of the infected cases will develop clinically significant disease. About one in 5-6 individuals will develop peptic ulcer disease during their lifetime, and <1% will develop gastric cancer^[4].

It is particularly important to recognize and define the characteristics of benign and pre-malignant lesions for application to studies of carcinogenesis and cancer prevention. Hence, in this study, we aimed to verify the occurrence of aneuploidies of chromosomes 3, 7, 8, 9, and 17, and of *TP53* gene deletions using the FISH technique, besides p53 expression, in benign lesions such as chronic gastritis, atrophic gastritis and gastric ulcer. The results were associated with *H pylori* infection and other clinicopathological features, such as sex, age, smoking and alcohol consumption.

MATERIALS AND METHODS

Samples

Chronic gastritis (CG), atrophic gastritis (CAG) and gastric ulcer (GU) samples were obtained, respectively, from 38 (13 males and 25 females), 13 (7 males and 6 females) and 21 patients (15 males and 6 females) without gastric cancer by biopsy performed during endoscopic evaluation. The mean age of these patients was 53 years (range, 2-89 years). Biopsies of histologically confirmed normal and *H pylori*-negative mucosa (NM) were obtained from 10 healthy individuals (2 males and 8 females) with a mean age of 43 years (range, 19-75 years). All specimens were collected at the Hospital de Base of São José do Rio Preto, SP, Brazil. All biopsies of normal mucosa and gastric ulcer were collected from the antral region of the stomach, whereas those of chronic gastritis and atrophic gastritis were mainly collected from the antrum and corpus. Clinicopathological data were collected using a standard interviewer-administered questionnaire and a review of the patients' medical records. The study was approved by the National Research Ethics Committee, and written informed consent was obtained from all subjects.

Histology

Hematoxylin-eosin (H&E) staining was used for the diagnosis and classification of gastritis^[5]. A modified Giemsa staining technique was used to visualize *H pylori*.

DNA extraction and PCR for *H pylori* diagnosis

DNA was extracted by the phenol-chloroform method after digestion with proteinase K^[6]. PCR assays were performed separately with approximately 100 ng of total DNA, using three different sets of oligonucleotides. One of them amplifies a 312-bp segment of the gene *CYP1A*^[7] as a DNA quality control, the other amplifies a 298-bp product of the gene encoding species-specific *H pylori* antigen^[8], and the last one amplifies a 411-bp fragment corresponding to the urease A gene^[9]. Positive and negative controls were used in all experiments. PCR products were separated on 75 g/L polyacrylamide-gel electrophoresis, followed by silver nitrate staining. The assay was considered positive when at least one of the bacterial PCR products was present^[10].

Fluorescence in situ hybridization (FISH)

Nuclear suspensions from all samples were obtained by mechanical and enzymatic disaggregation^[11]. After washes in 600 mL/L acetic acid, the resulting nuclear suspension was dropped onto microscope slides and stored at -70 °C until use for FISH assay.

FISH analysis was performed on interphase nuclei using centromere probes (CEP) for chromosomes 3, 7, and 8 directly labeled with Spectrum Green, and for chromosome 9 labeled with rhodamine (Vysis, Inc., Downers Grove, IL, USA). Dual-color FISH assays were performed using *TP53* probe kits (Vysis), including the LSI *TP53* sequence labeled in Spectrum Orange, with the centromere probe for chromosome 17 labeled in Spectrum Green. The hybridization and detection protocols followed the manufacturer's instructions.

For each probe, signals of about 300 intact and non-overlapping nuclei were evaluated by two independent observers according to the criteria described by Eastmond *et al*^[12]. Cutoff levels for aneuploidies were based on the upper-limit mean +4 SD of the normal gastric mucosa biopsies. By definition, for monosomy of chromosomes 3, 7, 8, 9, and 17, the cutoff values were set at 10.0%, 11.0%, 11.0%, 8.5%, and 7.5%, respectively, and, for trisomy of the same chromosomes, at 10.0%, 9.0%, 8.0%, 8.5%, and 7.0%, respectively. Tetrasomy and pentasomy were absent in the normal mucosa samples; thus, 2% was arbitrarily taken as cutoff value.

The *TP53* gene scores were taken together with those of the chromosome 17 centromere. For *TP53* gene deletion analysis, the gene-to-centromere ratio (G/C) was calculated for each case, that is, the total number of gene signals observed was divided by the total number of centromere signals.

Immunohistochemical analysis

To assess p53 protein accumulation, the commercially available monoclonal antibody DO-7 (Novocastra, Newcastle, UK) was used in combination with the anti-Ig second-stage antibody and the ABC kit (Novocastra) on paraffin-embedded sections. This antibody recognizes both mutant and wild types of p53 protein. The slides were counterstained with Harris hematoxylin. As positive controls, sections from gastric adenocarcinoma were processed, previously shown to express high levels of p53 protein. For each sample, 500 epithelial cells were counted.

Statistical analysis

Data were analyzed using χ^2 and Fisher's exact tests. *P* values less than 0.05 were considered statistically significant.

RESULTS

Analysis by the FISH technique showed that 89% to 99% of the nuclei of the *H pylori*-negative normal mucosa cases were disomic for chromosomes 3, 7, 8, 9, and 17, so they were considered to have no aneuploidy. The gene-to-centromere ratio (G/C) for the *TP53* gene deletion ranged from 0.97 to 0.99 (mean of 0.98), so, in the cases of benign lesions with a G/C ratio <0.90,

Table 1 Aneuploidy, *TP53* gene deletion and expression, and clinicopathological data in CG, CAG and GU cases

| Cases | Sex/age | Smoking/alcohol consumption | <i>H pylori</i> | L | <i>TP53</i> deletion (G/C<0.9) | p53 IHC (> 10%) | Aneuploidy |
|---------------------------|---------|-----------------------------|-----------------|---|--------------------------------|-----------------|---------------------------------|
| Chronic gastritis | | | | | | | |
| CG03 | M/32 | No/No | - | A | ND | ND | -7, +8 ^a |
| CG06 | M/02 | No/No | - | A | 0.97 | 15.6 | |
| CG13 | F/56 | Yes/No | + | C | 0.95 | 31.4 | |
| CG14 | F/36 | Yes/No | + | A | 0.94 | 0 | -7 |
| CG16 | M/89 | Yes/Yes | + | C | 0.98 | 12.0 | |
| CG19 | F/54 | No/Yes | - | A | 0.90 | 15.0 | +7 |
| CG30 | F/41 | Yes/No | + | A | ND | ND | +7 ^a |
| CG31 | M/44 | Yes/No | + | A | 0.95 | ND | +9 |
| CG33 | F/57 | No/No | + | A | 0.96 | 12.8 | |
| CG34 | F/34 | Yes/Yes | + | A | ND | ND | +7 |
| CG35 | M/54 | Yes/Yes | + | A | ND | ND | +3, +7, +17 |
| CG36 | F/58 | No/No | + | A | 0.96 | 0.8 | -7 |
| Atrophic gastritis | | | | | | | |
| CAG05 | F/84 | No/No | + | A | ND | ND | +8 ^a |
| CAG07 | M/65 | Yes/Yes | + | A | ND | ND | +7, +8 |
| CAG08 | M/57 | No/Yes | + | A | ND | ND | +7 / +7+7 |
| CAG13 | M/77 | Yes/No | + | A | ND | ND | +9 |
| Gastric ulcer | | | | | | | |
| GU02 | M/73 | Yes/Yes | - | A | ND | 0.8 | +17 |
| GU03 | M/52 | Yes/Yes | - | A | ND | 47.2 | +9 / +9+9 |
| GU04 | M/78 | Yes/Yes | - | A | ND | 9.6 | +7, +8 ^a |
| GU05 | M/64 | Yes/Yes | - | A | ND | 0.8 | -7 ^a |
| GU06 | M/43 | Yes/No | + | A | ND | 0 | +7 / +7+7, +8, -9, +17 / +17+17 |
| GU07 | F/51 | Yes/Yes | + | A | ND | 1.2 | +3 / +3+3 |
| GU09 | F/72 | No/No | + | A | 0.97 | 14.6 | |
| GU12 | M/28 | Yes/Yes | + | A | ND | 3.2 | -7, -9, -17 |
| GU14 | F/83 | No/No | + | A | ND | 1.2 | -9 |
| GU15 | M/43 | Yes/Yes | + | A | ND | 6.4 | -7, -8 |
| GU16 | F/82 | No/No | + | A | ND | 0 | -8 |
| GU18 | M/58 | Yes/Yes | + | A | ND | ND | +7 / +7+7 |
| GU19 | F/62 | Yes/No | + | A | ND | ND | +7 / +7+7, +8, +17 / +17+17 |
| GU20 | M/63 | Yes/Yes | + | A | ND | ND | +9 / +9+9 ^a |

M: male; F: female; L:location; -: *H pylori* negative; +: *H pylori* positive; A: antrum; C: corpus; ND: not done; ^a: chromosome 17 not analyzed.

gene deletion was assumed. Low-intensity nuclear p53 protein immunoreactivity was observed only in 4 of the 8 evaluated cases of normal gastric mucosa, ranging from 0.2% to 4.0% of the immunostained nuclei. The positivity index was defined as >10% of cells showing nuclear staining, according to a previous report by Gobbo-César *et al*^[11].

Table 1 shows the results regarding aneuploidies of chromosomes 3, 7, 8, 9, and 17, *TP53* gene deletion, p53 expression, and clinicopathological data only of those cases with benign lesions (chronic gastritis, atrophic gastritis and gastric ulcer) which showed some genetic alteration.

Increasing frequencies of aneuploidies were detected in the CG, CAG and GU cases: 21% (8/38), 31% (4/13), and 62% (13/21), respectively. The samples also showed an increasing complexity of the alterations with mainly monosomy and trisomy of chromosome 7 in CG, trisomy of chromosomes 7 and 8 in CAG, and more frequent trisomy of chromosomes 7, 8 and 17 in GU. About 75% (6/8) of CG, 100% (4/4) of CAG and 69% (9/13) of GU with aneuploidies were *H pylori*-positive. However, a significant association between the presence of

aneuploidies and *H pylori* infection was found only in CAG ($P=0.0143$) and GU ($P=0.0498$), while parameters such as sex, age, smoking and alcohol consumption did not show any association with the occurrence of aneuploidies.

No *TP53* gene deletion was found in the cases evaluated, whether in CG, in CAG or in GU, but low-intensity nuclear p53-positive immunoreactivity was present in 45% (5/11) and 12% (2/17) of the CG and GU cases, respectively, ranging from 12% to 47.2% of p53-positive nuclei, as compared to over-expression of the p53 protein in positive controls from gastric adenocarcinoma (Figure 1). No significant association was found either between p53 expression and *H pylori* infection or with the other clinicopathological features investigated.

DISCUSSION

The development of gastric carcinoma is considered as a multistage progressive process due to alterations in oncogenes, tumor suppressor genes, cell-adhesion molecules, telomerase, as well as to a genetic instability at several microsatellite loci, possibly related to *H pylori* infection^[13,14]. The mechanisms leading to mutations, hyperproliferation

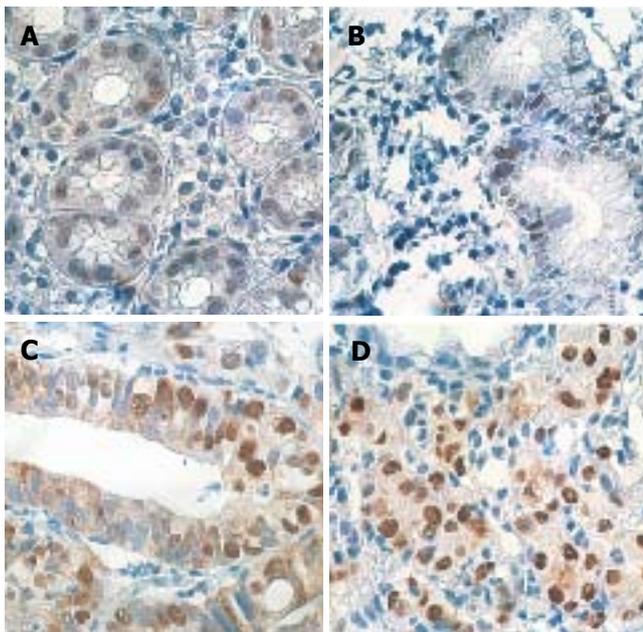


Figure 1 p53 protein expression on paraffin-embedded sections (400X original magnification). Low-intensity nuclear p53 protein immunoreactivity in chronic gastritis (A) and gastric ulcer (B) in comparison with p53 over-expression in the positive controls from gastric adenocarcinoma of intestinal type (C) and diffuse type (D).

and apoptosis of the epithelial cells are triggered very early in the *H. pylori* gastritis cascade, resulting in atrophic gastritis, intestinal metaplasia and gastric cancer^[2,13].

The identification of genetic alterations in biopsies of the epithelium of patients with gastric dyspepsia may be helpful in early diagnosis of gastric cancer. FISH is an accurate and sensitive technique for the detection of aneuploidies in interphase nuclei from biopsies, making it possible to identify changes in early stages of benign lesions, such as gastritis and gastric ulcer. Such changes may be used as markers for estimating the risk of gastric cancer.

A few studies have evaluated aneuploidy in epithelial lesions of the stomach using DNA flow cytometry. In chronic and atrophic gastritis, Weiss *et al*^[15] found DNA aneuploidy in 5.2% and in 8.5% of the cases, respectively. Teodori *et al*^[16] observed aneuploidy in 45% (9/20) chronic atrophic gastritis specimens, whereas Abdel-Wahab *et al*^[3] detected quantitative DNA alterations in 18.8% of atrophic gastritis cases.

To our knowledge, studies about aneuploidy in gastric ulcer are not available in the literature. Our study using the FISH technique showed increasing aneuploidy and complexity of the alterations from chronic gastritis (21%) towards atrophic gastritis (31%) and gastric ulcer (62%). The more frequent aneuploidies were monosomy and trisomy 7 in CG, trisomies 7 and 8 in CAG, and trisomies 7, 8, and 17 in GU. Moreover, in CAG and GU, these aneuploidies occurred more frequently in the *H. pylori*-positive cases, probably being responsible for the chromosomal instability observed.

Chromosomes 7, 8 and 17 carry several important genes involved in the control of cell proliferation, such as *EGFR* (7p12-13), *c-myc* (8q24), *HER-2/neu* (17q21), and *TP53* (17p13). Therefore, the presence of trisomy of these chro-

mosomes in precancerous gastric lesions leading to gene imbalance may confer proliferative advantage, increasing the risk of developing into gastric cancer.

The development of carcinoma in cases of gastric ulcer disease during long-term H2-blocker treatment is slowly increasing, and ulcers that require such treatment exhibit the characteristics of intractable conditions, including linear ulcers, simultaneous gastric and duodenal ulcers, immature intestinal metaplasia of the gastric epithelium, and atrophic gastritis accompanied by multiple ulcer cicatrices^[17]. Hence, especially in these cases of gastric ulcer, it is suggested that a genetic study should be performed to allow early detection of malignant transformation.

Inactivation of the *TP53* gene by mutation or deletion occurs as an initial event in gastric tumorigenesis, possibly associated with *H. pylori* infection^[14,18,19]. Using the FISH technique, Gomyo *et al*^[20] detected a *TP53* deletion in 77% (10/13) cases of gastric carcinoma.

In the present study, no *TP53* deletion was found in either chronic gastritis (15 cases), atrophic gastritis (3 cases) or gastric ulcer (3 cases), although it was observed in 60% (3/5) of the intestinal metaplasia and 110% (3/3) of the adenocarcinoma cases in a previous study conducted by our group^[11], thereby corroborating the hypothesis that these pathologies share genetic alterations and progress jointly.

The p53 gene products are known to regulate cell growth and proliferation. Wild-type p53 protein suppresses cell growth by controlling the G1 checkpoint, and has several additional physiological functions, including control of the G2 cell checkpoint, as well as mediation of apoptosis^[21]. The products of wild-type p53 can hardly be detected in normal cells by immunohistochemical assays because of their short half-life. However, *TP53* gene alterations induced by many missense mutations may modify the conformation and stabilize the proteins, leading to their accumulation^[22]. Therefore, over-expression of a p53 protein is a useful marker for detecting a mutated *TP53* gene^[23,24].

In this study, an increase of detectable low-intensity p53 was observed in 45% (5/11) of chronic gastritis and 12% (2/17) of gastric ulcer cases. These positive immunostaining findings may be a consequence of point mutations or small deletions. However, these changes are not detected by FISH, so were not tested in the current study. Alternatively, such alterations are more likely to be due to over-expression of the wild-type protein related to a physiological response brought about by the inflammatory process of the gastric epithelium. The lack of association between p53 expression and *H. pylori* infection may be related to the reduced number of specimens from benign lesions evaluated.

Although some studies have described p53 protein accumulation in benign gastric lesions^[13,25-28], its association with *H. pylori* infection in gastric carcinogenesis is not yet clear. Whereas some authors reported over-expression of p53 protein in *H. pylori*-positive chronic gastritis and intestinal metaplasia^[13,25,27,28], Craanen *et al*^[29] did not observe p53 protein accumulation in either atrophic gastritis or intestinal metaplasia and suggested that this process may be a late event in gastric carcinogenesis. On

the other hand, Lan *et al.*¹³ reported that *H pylori* infection might first cause severe imbalance of proliferation and apoptosis in the precancerous lesions, leading to p53-Rb tumor suppressor system mutation and telomerase reactivation, and finally causing gastric cancer.

In conclusion, our study has clearly shown the occurrence of aneuploidies, which increases with the progression of lesions from chronic gastritis to atrophic gastritis and to gastric ulcer, is frequently associated with *H pylori* infection, evidencing chromosomal instability in benign gastric lesions that may play an important role in gastric carcinogenesis. We observed no *TP53* gene deletion in the benign gastric lesions, whereas p53 protein overexpression was detected in some cases of chronic gastritis and gastric ulcer, thus suggesting that an accumulation of wild-type p53 protein occurs, probably due to an inflammatory response.

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RAPID COMMUNICATION

Cytotoxic T-lymphocyte antigen 4 gene polymorphisms and susceptibility to chronic hepatitis B

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Abstract

AIM: To assess the three polymorphism regions within cytotoxic T-lymphocyte antigen 4 (CTLA-4) gene, a C/T base exchange in the promoter region -318 (CTLA-4 -318C/T), an A/G substitution in the exon 1 position 49 (CTLA-4 49A/G), a T/C substitution in 1172 (CTLA-4 -1172T/C) in patients with chronic hepatitis B.

METHODS: Fifty-one patients with chronic hepatitis B virus infection and 150 healthy subjects were recruited sequentially as they presented to the hepatic clinic. Classification of chronic hepatitis B virus (HBV)-infected patients was as asymptomatic carrier state (26 patients) and chronic hepatitis B (25 patients). Genomic DNA was isolated from anti-coagulated peripheral blood Buffy coat using Miller's salting-out method. The presence of the CTLA-4 gene polymorphisms was determined using polymerase chain reaction amplification refractory mutation system (ARMS).

RESULTS: We observed a significant association between -318 genotypes frequency (T+C-, T+C+, T-C+) and susceptibility to chronic hepatitis B ($P=0.012$, OR=0.49, 95%CI: 0.206-1.162). However, we did not observe a significant association for +49 genotype frequency (T+C+, T+C- T-C+) and -1172 genotype frequency (C+T+, T+C- C+T-) and state of disease.

CONCLUSION: Our results suggest that CTLA-4 gene polymorphisms may partially be involved in the susceptibility to chronic hepatitis B.

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Key words: Cytotoxic T-lymphocyte antigen 4; Chronic hepatitis B; Gene polymorphism

INTRODUCTION

The human cytotoxic T-lymphocyte antigen 4 (CTLA-4) gene was mapped to chromosome 2q33^[1]. It consists of three exons; the first encodes a V-like domain of 116 amino acids. An A-to-G substitution at nucleotide 49 in exon 1 results in an amino acid substitution (Thr/Ala) in the leader peptide of the protein^[2]. The Ala allele has been shown to predispose the individual carrying it to the development of various immune diseases, including Graves' disease^[3], Hashimoto's thyroiditis^[4], Addison's disease, rheumatoid arthritis^[5,6], and celiac disease^[7,8]. The existence of inactive hepatitis B carriers with normal liver histology and function suggests that the virus is not directly cytopathic. The fact that patients with defects in cellular immune competence are more likely to remain chronically infected rather than to clear the virus is cited to support the role of cellular immune responses in the pathogenesis of hepatitis B-related liver injury. Differences in the robustness of cytolytic T-cell responsiveness and in the elaboration of antiviral cytokines by T cells have been invoked to explain the differences in outcomes between those who recover after acute hepatitis or between those with mild and those with severe acute hepatitis B virus infection^[9]. Chronic hepatitis is an important late complication of occult hepatitis B occurring in a small proportion of patients with chronic infection without having experienced an acute illness. Certain clinical and laboratory features suggest the progression of acute hepatitis to chronic hepatitis: (1) lack of complete resolution of clinical symptoms of anorexia, weight loss, and fatigue, and the persistence of hepatomegaly; (2) the presence of bridging or multilobular hepatic necrosis on liver biopsy during protracted, severe acute viral hepatitis; (3) failure of the serum aminotransferases, bilirubin and globulin levels to return to normal within 6-12 mo after the acute illness; and (4) the persistence of HBeAg beyond 3 mo after acute hepatitis. Under ordinary circumstances, none of the hepatitis viruses are known to be directly cytopathic to hepatocytes. Evidence suggests that the

clinical manifestations and outcomes after acute liver injury associated with viral hepatitis are determined by the immunologic responses of the host^[9]. It is thought that the majority of autoimmune endocrinopathies, including Graves' disease, autoimmune hypothyroidism, type 1 diabetes mellitus and autoimmune Addison's disease (sporadic as well as autoimmune polyendocrinopathy syndrome type 2), are inherited as complex genetic traits. Multiple genetic and environmental factors interact with each other to confer susceptibility to these disorders. In recent years, there have been considerable efforts towards defining susceptibility genes for complex traits. These investigations have shown, with increasing evidence, that the CTLA-4 gene is an important susceptibility locus for autoimmune endocrinopathies and other autoimmune disorders^[10]. Specific immunotherapy has moved into a new era with the introduction of soluble CTLA-4 protein into clinical trials. Treatment of bone marrow with CTLA-4 protein reduces rejection of the graft in HLA-mismatched bone marrow transplantation. In addition, promising results with soluble CTLA-4 have been reported in the down-regulation of autoimmune T-cell responses in the treatment of psoriasis^[9]. Treatment with a blocking antibody against the CTLA-4 gene has been shown to enhance the effect of tumor rejection in mice vaccinated with irradiated tumor cells^[11]. In this study, we have investigated whether genotypes of CTLA-4 -1172, -318, +49 polymorphisms as host factors predispose subjects to chronic hepatitis B.

MATERIALS AND METHODS

Subjects

Fifty-one patients with chronic hepatitis B virus infection (mean age, 36.23 ± 12.65 years) and 150 healthy subjects (mean age, 32.64 ± 7.12 years) were recruited sequentially as they presented to the hepatic clinic. Of chronic hepatitis B patients, 68.6% were males and 31.4% were females; and of healthy subjects (control group), 56% were males and 44% were females. Chronic HBV-infected patients were positive for HBV markers and if they had chronic hepatitis B, rising in alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity was seen for about 6 mo. Control groups were matched by age and sex; none of them were positive for hepatitis B serology. We did not regard any inflammatory conditions in the groups. All studies were carried out after the approval of Local Hospital Ethical Committees.

Hepatitis B was regarded if positive for HBsAg, anti-HBe, anti-HBc by ELISA HBsAg+, HBeAg (+, -), anti-HBe (+, -), HBV DNA (+, -), IgG anti-HBc+. Classification of chronic HBV-infected patients was carried out as follows: (A) Asymptomatic carrier state (26 patients), composed of chronically HBsAg-positive patients who had anti-HBc in serum, anti-HBs either undetected or detected at low titer against the opposite subtype specificity of the antigen regarded as inactive or asymptomatic carriers, HBV DNA load <10⁵ copies/mL, HBeAg (+, -), liver transaminases of normal range; (B) chronic hepatitis B (25 patients) which comprised, based upon biochemical, virologic and histological activity, 17 patients who were still

on interferon or lamivudine treatment, 5 non-responders to treatment (lack of virologic and or histologic response by first treatment course in whom sustained response was unlikely) and 3 chronic hepatitis B patients. Histologic classification was measured by modified histologic activity index (HAI) by Ishak score.

Treatment protocol

Management of chronic hepatitis B directed at suppressing the level of virus replication for patients who had HBV DNA detectable by hybridization assay and histological evidence of chronic hepatitis on liver biopsy. Interferon- α (IFN- α) or lamivudine was used for treatment. IFN- α registered as a 16-wk course was given subcutaneously at a daily dose of 5 million units or thrice a week at a dose of 10 million units. Lamivudine was administered orally at a daily dose of 100 mg for 12 mo. No treatment was recommended for inactive "non-replicative" hepatitis B carriers. Control subjects had no evidence of hepatitis B infection.

DNA isolation

Ten milliliters of venous blood was collected from each subject into tubes containing 50 mmol/L EDTA, and genomic DNA was isolated from anti-coagulated peripheral blood Buffy coat using Miller's salting-out method. All samples from cases and controls were handled in identical fashion as previously described^[13].

Analysis of CTLA-4 gene polymorphisms

The presence of the CTLA-4 gene polymorphisms was determined by polymerase chain reaction (PCR) amplification refractory mutation system as previously described^[6].

Statistical analysis

Allele and genotype frequencies among cases and controls were compared with values predicted by the Hardy-Weinberg equilibrium using the χ^2 test. All probability values were two-tailed. Distributions of alleles in patients and controls were compared using Fisher's exact test. The difference between susceptibility to hepatitis B was analyzed using Student's *t* test. A *P* value less than 0.05 was considered statistically significant. SPSS software version 11.05 was used for analysis.

RESULTS

Of chronic hepatitis B group, 26 cases (12.9%) were asymptomatic carriers, 3 cases (1.5%) were chronic hepatitis B, 17 cases (8.5%) were chronic hepatitis B on first course of treatment, and 5 cases (2.5%) were non-responders to first course of treatment. In addition, 86.3% of chronic hepatitis B patients were HBeAg-negative. No significant difference was observed between the mean age of HBeAg-positive patients (34.8 years) and HBeAg-negative patients (36.4 years). Of the 44 HBeAg-negative chronic hepatitis B patients, 25 cases were inactive carriers, 12 cases had received treatment, 4 cases were non-responders and 3 patients were still on treatment (*P* < 0.0001).

Distribution of genotype frequency of CTLA-4 +49,

Table 1 Distribution of allele and genotype frequency of CTLA-4 +49, -318, -1172 polymorphisms in hepatitis B patients and controls

| | Chronic hepatitis B <i>n</i> (%) | Controls <i>n</i> (%) |
|--|-------------------------------------|--------------------------|
| +49 genotype frequency ($P=0.211$) | | |
| G+A- | 9 (17.6) | 41 (27.3) |
| G+A+ | 16 (31.4) | 52 (34.7) |
| G-A+ | 26 (51) | 57 (38) |
| +49 allele frequency | | |
| G+ | 25 (49) | 93 (62) |
| A+ | 42 (82.4) | 109 (72.7) |
| -318 genotype frequency ($P=0.012$) | | |
| T+C- | 0 (0) | 6 (4) |
| T+C+ | 10 (19.6) | 10 (6.7) |
| T-C+ | 41 (80.4) | 134 (89.3) |
| -318 allele frequency | | |
| T+ | 10 (19.6) | 16 (10.7) |
| C+ | 51 (100) | 44 (96) |
| -1172 genotype frequency ($P=0.061$) | | |
| T+C- | 36 (70.6) | 108 (72) |
| T+C+ | 13 (25.5) | 42 (28) |
| T-C+ | 2 (3.9) | 0 (0) |
| -1172 allele frequency | | |
| C+ | 15 (29.4) | 42 (28) |
| T+ | 49 (96.1) | 150 (100) |

-318, -1172 polymorphisms of hepatitis B patients and controls is shown in Table 1. We observed a significant association between genotype frequency of -318T⁺C⁺ genotype and hepatitis B disease (19.6% *vs* 6.7%, $P=0.012$). On the contrary, no obvious difference was observed between HBeAg positive or negative hepatitis B disease and frequency of either studied genotypes. Moreover, we found a significant association between -318 genotypes frequency (T+C-, T+C+, T-C+) and susceptibility to chronic hepatitis B ($P=0.012$, OR=0.490, 95%CI: 0.206-1.162).

There was no other association between genotype frequency of other genotypes frequencies and hepatitis B disease. Mean AST and ALT activities were 32.66 ± 19.71 and 38.21 ± 27.42 IU/L (normal range, 5-30 U/L), respectively. We observed significant differences in the mean AST levels among the inactive carriers (21.34 IU/L), chronic hepatitis B on treatment (37.76 IU/L), chronic hepatitis B who had received treatment (74.4 IU/L), and non-responders to treatment (31 IU/L) ($P < 0.0001$). Moreover, significant differences were observed in the mean ALT levels among the inactive carriers (22.34 IU/L), chronic hepatitis B on treatment (45.7 IU/L), chronic hepatitis B who had received treatment (93 IU/L), and non-responders to treatment (42 IU/L) ($P < 0.0001$). The relation between frequency of genotypes of CTLA-4 49, -318, -1172 CTLA-4 polymorphisms and mean AST and ALT levels in subjects with chronic hepatitis, chronic hepatitis on first course of treatment, inactive carrier, and non-responders to first course of treatment is shown in Table 2.

Percutaneous liver needle biopsy was performed in 24 cases. Mean pathologic grade and stage were 5.88 ± 1.84

Table 2 Relation between frequencies of genotypes of CTLA-4 +49, -318, -1172 CTLA-4 polymorphisms and mean AST and ALT levels in subjects with chronic hepatitis, chronic hepatitis on first course of treatment, inactive carrier, non-responders to first course of treatment

| | TC 1172 genotype | Mean | SE |
|--|-------------------------|-------------|-----------|
| AST | 1172 T+C- | 35.1944 | 3.7008 |
| | 1172 T+C+ | 25.3077 | 2.3325 |
| | 1172 T-C+ | 35.0000 | 14.0000 |
| | TC 318 genotype | Mean | SE |
| AST | 318 T+C+ | 29.1000 | 5.8299 |
| | 318 T-C+ | 33.5366 | 3.1455 |
| There are no valid cases for AST. Statistics cannot be computed. | | | |
| | AG 49 genotype | Mean | SE |
| AST | 49 G+A- | 32.6667 | 6.5362 |
| | 49 G+A+ | 32.3750 | 3.9811 |
| | 49 G-A+ | 32.8462 | 4.3960 |
| | TC 1172 genotype | Mean | SE |
| ALT | 1172 T+C- | 40.9167 | 5.0528 |
| | 1172 T+C+ | 28.9231 | 4.3742 |
| | 1172 T-C+ | 50.0000 | 20.0000 |
| | TC 318 genotype | Mean | SE |
| ALT | 318 T+C+ | 33.5000 | 7.0683 |
| | 318 T-C+ | 39.3659 | 4.4751 |
| There are no valid cases for ALT. Statistics cannot be computed. | | | |
| | AG 49 genotype | Mean | SE |
| ALT | 49 G+A- | 43.1111 | 9.3592 |
| | 49 G+A+ | 40.1875 | 5.7018 |
| | 49 G-A+ | 35.3077 | 5.9497 |

and 2.11 ± 1.13 , respectively and mean score was 7.9 ± 2.48 . Mean histology score for 1172 T+C- genotype was 7.68 (95%CI: 6.4-8.9), for 1172 T+C+ genotype was 8.75 (95%CI: 4.9-12.5), for -318 T+C+ genotype was 8.25 (95%CI: 6.7-9.7), for -318 T-C+ genotype was 8.75 (95%CI: 6.5-9.11), for 49 A+G+ genotype was 8 (95%CI: 5-10.9), and for 49 A+G+ genotype was 7.7 (95%CI: 5.2-10.3).

Association between CTLA-4 +49, -318, -1172 polymorphisms, and state of disease in chronic hepatitis B patients is shown in Table 3. We observed a significant association between +49 genotypes (G+A-, G+A+, G-A+), and state of disease in chronic hepatitis B patients ($P=0.043$). In contrast, no significant relation between -318 genotype frequency (T+C+, T+C-, T-C+), -1172 genotype frequency (C+T+, T+C-, C+T-) and state of disease was observed.

DISCUSSION

In a study^[14] to test the hypothesis that single-nucleotide polymorphisms (SNPs) in the gene encoding CTLA-4 may affect the vigor of the T-cell response to hepatitis B virus (HBV) infection and thus influencing viral persistence, they studied genotyped six CTLA-4 SNPs, from which all frequent haplotypes can be determined, using a large, matched panel of subjects with known HBV outcomes. Haplotypes with these SNPs were constructed for each subject using PHASE software. The haplotype distribution

Table 3 Association between CTLA-4 +49 genotype polymorphism and state of disease in chronic hepatitis B patients

| | | | State of disease | | | | Total |
|-----------------|----------|---------------------------|------------------|-----------------|-------------------------|-------------------|--------|
| | | | Healthy carrier | Under treatment | Refractory to treatment | Chronic hepatitis | |
| AG +49 genotype | +49 G+A- | Count | 5 | 2 | 2 | 0 | 9 |
| | | % within state of disease | 19.2% | 11.8% | 40.0% | .0% | 17.6% |
| | +49 G+A+ | Count | 6 | 7 | 0 | 3 | 16 |
| | | % within state of disease | 23.1% | 41.2% | .0% | 100.0% | 31.4% |
| | +49 G-A+ | Count | 15 | 8 | 3 | 0 | 26 |
| | | % within state of disease | 57.7% | 47.1% | 60.0% | .0% | 51.0% |
| Total | | Count | 26 | 17 | 5 | 3 | 51 |
| | | % within state of disease | 100.0% | 100.0% | 100.0% | 100.0% | 100.0% |

¹*P*=0.043.

differed between those with viral persistence and those with clearance. Two haplotypes were associated with the clearance of HBV infection, which were most likely due to the associations with the SNPs -1722C (OR=0.60, *P*=0.06) and +49G (OR=0.73, *P*=0.02). The wild-type haplotype, which contains a SNP leading to a decreased T-cell response (+6230A), was associated with viral persistence (OR=1.32, *P*=0.04). These data suggest that CTLA-4 influences recovery from HBV infection, which is consistent with the emerging role of T regulatory cells in the pathogenesis of disease. The results of the present study showed a difference in CTLA-4 -318 genotype distribution between subjects and controls. In this study, we have demonstrated a significant association between -318 genotypes frequency (T+C-, T+C+, T-C+) and susceptibility to chronic hepatitis B (*P*=0.012), whereas there was no significant relation between -318 genotype frequency (T+C+, T+C-, T-C+) and -1172 genotype frequency (C+T+, T+C-, C+T-) and state of disease.

Chronic hepatitis B is either HBeAg positive or negative. In the population studies, HBeAg-negative (anti-HBe-reactive) was found more common than HBeAg-reactive chronic hepatitis B [44 (86.3%) *vs* 7 (13.7%), *P*=0.082]. In this study population, mean levels of both AST and ALT were significantly higher in HBeAg-positive patients as compared with HBeAg-negative patients. HBeAg-negative chronic hepatitis B patients tend to have progressive liver injury, complicated more frequently by cirrhosis and hepatocellular carcinoma, episodic reactivation of liver disease and more refractory to antiviral therapy. Most such cases represent precore or core-promoter mutations. Patients with HBeAg-negative phenotype or precore mutants are unable to secrete HBeAg and tend to have severe liver disease^[9]. Precore genetic mutant of HBV is associated with the more severe outcome of HBV infection; therefore, relative pathogenicity is a property of the virus, and not the host^[9]. In this study, we did not regard the influence of viral effects, such as hepatitis D virus, hepatitis B virus DNA load and YMDD variants, on the susceptibility and outcome of hepatitis B. However, as most of our patients were HBeAg-negative, this predominance was seen in all states of disease composed of asymptomatic carriers, subjects still on treatment, non-responders and subjects who finished treatment.

In other studies, especially those discussing about autoimmune diseases, a greater association between polymorphisms of this gene and disease has been demonstrated^[15-18]. The strong genetic associations so far in PBC are with chromosomes 6p21.3 and 2q and include HLA DRB1*08 haplotypes, CTLA-4 G and IL1RN-IL1B haplotypes, CASP8, and nramp1. It is unlikely that only genes that influence disease susceptibility and progression in primary biliary cirrhosis (PBC) are immunoregulatory genes concerned with T-cell immunity. Recent studies have indicated a new era for immunogenetics, when genes encoding all immune active proteins may be considered as candidates. One should not concentrate solely on the immune response as recent investigations of mannose binding lectin and apolipoprotein-E have been testified. The authors stated the key issues for future investigators include defining the mechanisms whereby self tolerance is broken, defining the mechanisms that determine the rate of disease progression, and identifying genetic markers to predict progression and malignancy, and assessing the genetic basis of variability in disease progression. The significant variation in the rate of progression of PBC has led to the hypothesis that genes, in addition to contributing to disease susceptibility, may also determine the rate of disease progression. Several of the studies mentioned earlier have suggested associations between alleles at polymorphic loci and rate of progression. One problem inherent in such studies is that of definition of disease progression^[15]. PBC is an autoimmune cholestatic liver disease thought to be developed through a complex interaction of genetic and environmental factors. It is characterized by T-cell-mediated non-suppurative destructive cholangitis. We have studied the polymorphic CTLA-4 gene, which encodes a molecule that is a vital negative regulator of T-cell activation, as a candidate susceptibility locus for PBC. This gene on chromosome 2q33 (designated IDDM12) is associated with susceptibility to both type 1 diabetes and autoimmune thyroid disease. The CTLA-4 exon 1 polymorphism (A/G encoding for threonine or alanine, respectively) was genotyped via PCR in 200 Caucasoid PBC patients and 200 non-related geographically matched Caucasoid controls. There was a significant over-representation of the G/A and G/G genotypes in PBC patients as compared with controls. Likewise, there was a significant difference in

allele frequencies. This association remained significant ($P=0.00027$) when patients with autoimmune thyroid disease were excluded from the analysis. Thus, it was concluded that the CTLA-4 exon 1 polymorphism is the first non-major histocompatibility complex gene to be identified as a susceptibility locus for PBC. The data support the hypothesis that clinically distinct autoimmune disease may be controlled by a common set of susceptibility genes^[16].

Latent autoimmune diabetes in adults (LADA) is identified by the presence of GAD65 autoantibodies in diabetic patients who do not require insulin treatment for at least 6 mo after the diagnosis. Previous studies have shown that the risk for LADA, similarly to type 1 diabetes mellitus (T1DM), is increased in subjects carrying the HLA-DRB1*03-DQA1*0501-DQB1*0201 and/or HLA-DRB1*04-DQA1*0301-DQB1*0302 haplotypes. Moreover, it has been investigated the association between LADA and the CTLA-4 A/G polymorphism, another gene polymorphism associated with T1DM and other autoimmune diseases. The heterozygous A/G genotype was significantly more frequent among 80 LADA (69%) than among 85 healthy subjects of similar age and geographical provenience (47%) (OR = 2.47, corrected $P=0.023$). Conversely, the homozygous A/A genotype was significantly less frequent in LADA subjects compared to healthy controls (26% vs 47%, OR = 0.4, corrected $P=0.028$). The results of our study showed that LADA was positively associated with the CTLA-4 A/G genotype, similarly to T1DM, thus providing further supporting evidence of the autoimmune origin of this form of diabetes mellitus in adults^[17]. The CTLA-4 molecule plays an important role in immune regulation by down-regulating activation of T cells by antigen-presenting cells. Polymorphisms of the CTLA-4 gene have been shown to be associated with susceptibility to a number of autoimmune diseases. Some, but not all, studies suggest the association between the CTLA-4 gene and autoimmune hypothyroidism. The aim of this study was to determine whether allelic association was present between the A-G SNP at position 49 in exon 1 of the CTLA-4 gene and autoimmune hypothyroidism. The study was performed in 158 patients with autoimmune hypothyroidism and 384 control subjects. All subjects were white Caucasians from UK. There was a significant excess of the G allele in patients with autoimmune hypothyroidism as compared with controls. The GG and the AG genotypes were found to be more frequent in patients with autoimmune hypothyroidism than controls. These results suggested that the CTLA-4 gene region on chromosome 2q33 is a susceptibility locus for autoimmune hypothyroidism^[18].

In conclusion, in the population that was studied, there is a significant difference in the frequencies of the -318 CTLA-4 gene polymorphisms (T⁺C⁻, T⁺C⁺, T⁻C⁺) and susceptibility to chronic hepatitis B between controls and patients with hepatitis B. This association supports that this gene is partially the operating mechanism for genetic susceptibility to hepatitis B disease.

However, current knowledge of the genetic basis of susceptibility to hepatitis B is incomplete. Further investigations should be considered with caution until

it is confirmed in independent large series. Elucidating predisposing genetic associations will markedly assist in understanding the susceptibility and pathophysiology of disease, the possibility of identifying the susceptibility of patients who are at elevated risk to hepatitis B infection or are in the early stages of disease or more rapid progression course of disease, would have obvious clinical benefit in terms of patient management and therapy.

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RAPID COMMUNICATION

Effect of garlic on isoniazid and rifampicin-induced hepatic injury in rats

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Abstract

AIM: To evaluate the hepatoprotective effect of garlic on liver injury induced by isoniazid (INH) and rifampicin (RIF).

METHODS: Wistar rats weighing 150-200 g were treated orally with 50 mg/kg of INH and RIF daily each for 28 d. For hepatoprotective studies, 0.25 g/kg per day of freshly prepared garlic homogenate was administered orally half an hour before the INH+RIF doses. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and bilirubin were estimated on d 0, 14, 21, and 28 in all the rats. Histological analysis was carried out to assess the injury to the liver. Lipid peroxidation (LPO) as a marker of oxidative stress and non-protein thiols (glutathione) for antioxidant levels were measured in liver homogenate.

RESULTS: The treatment of rats with INH+RIF (50 mg/kg per day each) induced hepatotoxicity in all the treated animals as judged by elevated serum ALT, AST, and bilirubin levels, presence of focal hepatocytic necrosis (6/8) and portal triaditis (8/8). Garlic simultaneously administered at a dose of 0.25 g/kg per day prevented the induction of histopathological injuries in INH+RIF co-treated animals, except in 4 animals, which showed only moderate portal triaditis. The histological changes correlated with oxidative stress in INH+RIF treated animals. The group which received 0.25 g/kg per day garlic homogenate along with INH+RIF showed higher levels of glutathione ($P < 0.05$) and low levels of LPO ($P < 0.05$) as compared to INH+RIF treated group.

CONCLUSION: Freshly prepared garlic homogenate protects against INH+RIF-induced liver injury in experimental animal model.

INTRODUCTION

Tuberculosis (TB) is one of the most common infectious diseases. In India, pulmonary tuberculosis is one of the major causes for adult deaths^[1]. INH and RIF, the first line drugs used for tuberculosis chemotherapy, are associated with hepatotoxicity^[2]. The rate of hepatotoxicity has been reported to be much higher in developing countries like India (8%-30%) compared to that in advanced countries (2%-3%) with a similar dose schedule^[3]. We have established in our laboratory oxidative stress as one of the mechanisms for INH+RIF-induced hepatic injury^[4]. Majority of normally formed free radicals are removed by the action of reduced glutathione. In circumstances where there is a reduction in glutathione results in the initiation of lipid peroxidation (LPO) resulting in tissue injury^[5]. Garlic, an antioxidant, has been shown to inhibit LPO^[6] and dose-dependent induction of endogenous antioxidants in rat kidney and liver^[7]. Hence this protocol was designed to study the protective effect of garlic on INH+RIF-induced liver injury in Wistar rats.

MATERIALS AND METHODS

Materials

Wistar rats weighing 150-200 g body were used in the study. The protocol was approved by the Institute's Animal Ethical Committee. Body weights of these rats were monitored sequentially in control and experimental animals for a period of 28 d. Animals were divided into four groups as control ($n=8$), INH+RIF ($n=8$), garlic ($n=8$), and INH+RIF+garlic ($n=12$) where n was the number of animals included in the study.

For hepatotoxic model, 50 mg/kg per day of INH and RIF each was used in the study^[8]. INH and RIF solutions were prepared separately in sterile distilled water. The pH of RIF solution was adjusted to 3.0 with 0.1 mol/L HCl^[9].

INH and RIF were administered orally for 28 d. Liver transaminases and bilirubin were estimated on d 0, 14, 21, and 28 in both control (saline treated) and experimental animals. The criteria for hepatotoxicity were the presence of histological changes such as hepatocytic necrosis and portal triaditis along with elevated transaminases (more than thrice the upper normal limit). For the hepatoprotective model, 0.25 g/kg per day of freshly prepared garlic homogenate along with INH+RIF solution was administered^[10].

For the preparation of fresh garlic homogenate, garlic bulbs were purchased from a local supermarket, cut into small pieces and homogenized in a motor-driven Teflon glass homogenizer on ice.

Methods

Rats were killed after the INH+RIF and garlic treatment. Representative blocks of liver were taken and fixed in 40g/L formaldehyde. Light microscopic examination of the liver was done on sections stained with (H) and (E). Special stains like reticulin and Ziehl Nielsen for acid fast bacilli were carried out whenever necessary. Various slides of different groups (two slides per animal) containing full cross-sections of the major hepatic lobules were prepared.

Twelve hours after the last oral treatment, rats were killed by cervical dislocation. Liver was excised immediately, quickly cooled and perfused with cold normal saline. Ten percent homogenate was prepared by homogenizing the liver tissue in 100 mmol/L potassium phosphate, 150 mmol/L potassium chloride buffer containing 200g/L glycerol (pH 7.5). LPO in tissue homogenate was measured by the reaction of LPO products like malondialdehyde (MDA) with thiobarbituric acid by the method of Kornbrust and Mavis^[11].

Estimation of thiols (non-protein thiols) was carried out in tissue homogenate according to the method of Sedlak and Lindsay^[12]. Protein estimation was done by the method of Lowry *et al.*^[13].

Statistical analysis

Data were analyzed by analysis of variance (ANOVA) followed by multiple comparisons using Dunnett's procedure to compare all groups against control, Student-Newman-Keuls procedure was used to compare all the groups' pair wise.

RESULTS

There was no mortality in any of the groups. The body weight and relative liver weights of the experimental animals calculated at the end of the study had no statistically significant difference when compared to the control animals (Table 1).

Three-fold rise above the normal upper limits in the measured serum transaminases in INH+RIF group on d 28 of the experiment was a biochemical indication of liver injury. Garlic-treated and control animals had normal values of transaminase (Table 2). However, there was a significant increase in serum bilirubin in all the animals of INH+RIF group (Table 2) and in four animals of INH+RIF+garlic group in whom histological changes

Table 1 Body weight and relative liver weights of control and the rats treated with INH + RIF, garlic and INH + RIF + garlic (mean±SD)

| Treatment (n) | Mortality (dead/total) | Body weight (g) | Relative liver weight (g) |
|---------------------|------------------------|--------------------|---------------------------|
| Control (8) | 0/8 | 153±29 | 4.65±0.32 |
| INH+RIF (8) | 0/8 | 150±3 ¹ | 4.27±0.41 |
| Garlic (8) | 0/8 | 148±3 ¹ | 4.51±0.40 |
| INH+RIF+garlic (12) | 0/12 | 159±3 ¹ | 4.69±0.29 |

No significantly different among group.

Table 2 Liver function tests in rats treated with INH + RIF, garlic and INH + RIF + garlic at 4 wk (mean±SD)

| Treatment (n) | ALT (IU/L) | AST (IU/L) | Bilirubin mg/dL |
|---------------------|---------------------------|----------------------------|---------------------------|
| Control (8) | 30.75±7.04 | 138±6.39 | 0.498±0.157 |
| INH+RIF (8) | 108.8±20.06 ^b | 472±85.9 ^b | 2.52±0.355 ^b |
| Garlic (8) | 29.25±5.65 | 130.8±9.3 | 0.621±0.117 |
| INH+RIF+garlic (12) | 56.8±33.05 ^{a,b} | 225.8±130.3 ^{a,b} | 1.01±0.566 ^{a,b} |

^a*P*<0.05 vs INH + RIF; ^b*P*<0.01 vs Control and Garlic.

Table 3 Lipid peroxidation and non-protein thiols in control, INH + RIF, garlic and INH + RIF + garlic groups (mean±SD)

| Treatment (n) | Lipid peroxidation (MDA/g tissue/min) | Non-protein thiols (μmol/g tissue) |
|---------------------|---------------------------------------|------------------------------------|
| Control (8) | 10.4±1.5 | 3.8±0.5 |
| INH+RIF (8) | 16.5±1.9 ^b | 1.6±0.4 ^b |
| Garlic (8) | 9.3±2.6 | 4.0±0.6 |
| INH+RIF+garlic (12) | 13.2±1.6 ^{a,b} | 2.7±0.6 ^{a,b} |

^a*P*<0.05 vs INH + RIF; ^b*P*<0.01 vs Control and Garlic.

were present. Serum bilirubin was not significantly different in control, garlic and INH+RIF+garlic groups in whom histological changes were not present at the end of the study.

The treatment of rats with INH+RIF (50 mg/kg per day each) induced hepatotoxicity in all the treated animals as judged by elevated serum ALT, AST, and bilirubin levels, portal triaditis (8/8) and liver necrosis (Figure 1A and 1B). Simultaneously administered garlic at a dose of 0.25 g/kg per day prevented the induction of histopathological injuries in INH+RIF co-treated animals (8/12), except in four animals, who showed only moderate portal triaditis (Figure 1C).

The INH+RIF-administered animals exhibited significantly low levels of hepatic non-protein thiols (*P*<0.01) as compared to control (Table 3) and co-administration of garlic and INH+RIF showed increased levels in hepatoprotected group as compared to control (*P*<0.01). Fresh garlic homogenate by itself did not affect hepatic non-protein thiols significantly as compared to control. Treatment with INH+RIF depleted significantly the content of non-protein thiols as compared to control (*P*<0.001, Table 3). Co-administration of freshly prepared garlic homogenate maintained its levels in the liver protected group although less than that in the control group (*P*<0.05).

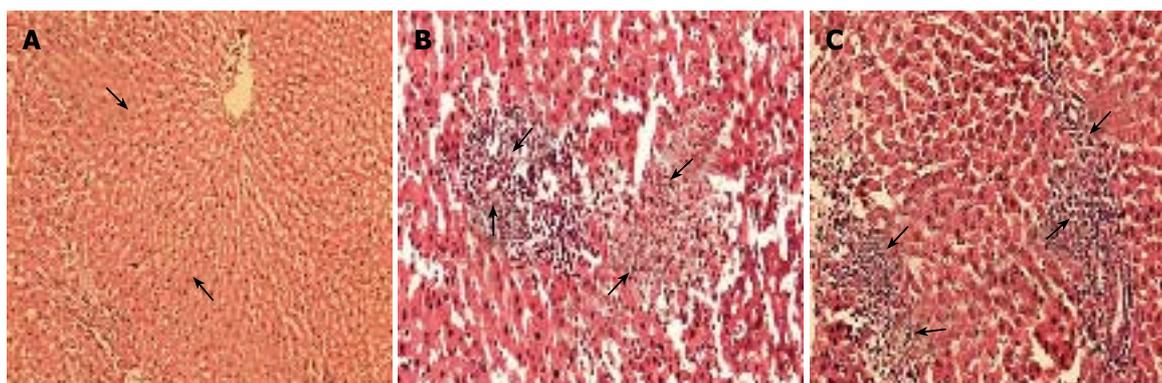


Figure 1 Normal morphology (A), hepatocytic necrosis (B) and portal triaditis (C) in control, INH+RIF and INH+RIF+garlic groups (H E \times 55).

The treatment with INH+RIF significantly enhanced the peroxidation of lipids ($P < 0.01$) and co-administration of freshly prepared garlic homogenate blocked the induction of LPO caused by INH+RIF treatment. Freshly prepared garlic homogenate-treatment alone did not reduce the LPO levels (Table 3) in rats.

DISCUSSION

In the present study, hepatotoxicity model in Wistar rats was successfully produced by administering INH and RIF (50 mg/kg per day each) orally. The protective effect of 0.25 g/kg per day freshly prepared garlic homogenate on INH+RIF induced liver injury in rats was evaluated as previously described^[8]. We did not study the metabolites of administered dose of garlic in the blood of INH+RIF-treated rats because absorption and metabolism of alliin-derived compounds are only partially understood. Alliin and alliin-derived compounds, including diallyl sulfides (DAS), ajoene and vinylthiins have never been detected in human blood, urine or stools even after the consumption of fresh garlic up to 25 g or 60 mg of pure alliin^[14].

Garlic bulb contains approximately 65% water, 28% carbohydrates (mainly fructans), 2.3% organosulfur compounds, 2% protein (mainly alliinase), 1.2% free amino acids (mainly arginine) and 1.5% fiber. Intact garlic bulbs contain a high amount of γ -glutamylcysteine. These reserve compounds can be hydrolyzed and oxidized to form alliin, which accumulates naturally during storage of garlic bulbs at cool temperature. After various kinds of processing, such as cutting, crushing, chewing or dehydration, the vacuolar enzyme, alliinase, rapidly lyses cytosolic cysteine sulfoxides (alliin) to form the odoriferous alkyl alkane-thiosulfinates such as alliin. Alliin and other thiosulfinates instantly decompose to other compounds, such as DAS, diallyl disulfide (DADS), and diallyltrisulfide (DATS), dithiins and ajoene. At the same time, γ -glutamylcysteines are converted to S-allyl cysteine (SAC) via a pathway other than the alliin/alliin pathway^[15].

Garlic has been found to have an important dietary and medicinal role for centuries. Most of its prophylactic and therapeutic effects are ascribed to specific oil and water soluble organosulfur compounds. Thiosulfinates and other secondary metabolites of garlic, including

steroids, terpenoids, flavonoids and other phenols, may be responsible for reported therapeutic effects of garlic. Reuter *et al*^[16] have reviewed the therapeutic effects of garlic on cardiovascular system as well as its antibiotic, anticancer, antioxidant, immunomodulatory, anti-inflammatory, hypoglycemic and hormone-like effects. Garlic also increases anti-inflammatory monocyte IL-10 production and decreases proinflammatory cytokines such as TNF- α , IL-1 β , IL-6, IL-8, T cell interferon gamma, IL-2^[17].

Garlic, a natural substance, has also been shown to inhibit LPO^[18]. Phytochemicals from plant rich diets (including garlic) provide an important additional protection against oxidative damage^[19]. There are a variety of antioxidants in garlic, which protect against disease-causing oxidative damage^[20]. Garlic and related organosulfur compounds have antioxidant, detoxifying and other properties. These detoxifying effects are related to their ability to inhibit phase I enzymes and induce phase II enzymes or bind to exogenous toxins through sulfhydryl groups^[21]. Previous studies on the mechanism of INH+RIF-induced hepatotoxicity have shown that non-protein thiols play a very important role in the detoxification of reactive toxic metabolites of INH+RIF. Liver injury has been observed when glutathione stores are markedly depleted^[22]. The present study has further strengthened the protective role of garlic in INH+RIF-induced hepatic non-protein thiol depletion. These observations may be due to the inhibition of bioactivation of INH+RIF metabolites resulting in the decreased formation of INH electrophiles. A similar protective role of garlic has been documented in acetaminophen-induced hepatotoxicity^[23].

Non-protein thiol is an important defense mechanism in living cells. As a substrate for antioxidant enzymes, i.e. glutathione peroxidase and glutathione reductase, it protects cellular constituents from the damaging effects of peroxidase formed in metabolism and other reactive oxygen species reactions. Aged garlic extract increases cellular glutathione in a variety of cells including those in normal liver and mammary tissue^[24]. In the present study, the oxidative injury induced by INH and RIF could be prevented by fresh garlic homogenate. Thus this study represents a novel and an attractive idea to prevent INH+RIF-induced hepatic injury by co-administration of fresh garlic homogenate.

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RAPID COMMUNICATION

Clinical outcome of intersphincteric resection for ultra-low rectal cancer

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Abstract

AIM: To analyze oncological outcome of intersphincteric resection (ISR) in ultra-low rectal cancer with intent to spare colostoma.

METHODS: From 1995 to 1998, patients with a non-fixed rectal adenocarcinoma (tumor stage T2) preserving the lower margin at 1-3 cm above the dentate line without distant metastasis was enrolled (period I). ISR was practiced in eight patients, and their postoperative follow-up was at least 5 years. In addition, from 1999 to 2003, another 10 patients having the same tumor location as period I underwent ISR (period II). Among those, 6 patients with T3-4-staged tumor received preoperative chemoradiotherapy.

RESULTS: All patients received ISR with curative intention and no postoperative mortality. In these case series at period I, local recurrence rate was 12.5% and metastasis rate 25.0%; the 5-year survival rate was 87.5% and disease-free survival rate 75.0%. There was no local recurrence or distant metastases in 10 patients with a median follow-up of 30 (range, 18-47) mo at period II.

CONCLUSION: As to ultra-low rectal cancer, intersphincteric resection could provide acceptable local control and cancer-related survival with no permanent stoma in early-staged tumor (tumor stage T2); moreover, preoperative concurrent chemoradiotherapy would make ISR feasible with surgical curative intent in more advanced tumors (tumor stages T3-4).

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INTRODUCTION

In earlier ages, tumors within 7-8 cm above the anal verge were treated by abdominoperineal resection (APR), especially when the rectal tumor could be easily palpated by an examiner's finger.

Along with the recent development of circular stapling devices, it facilitates the feasibility of ultra-low anterior resection of rectal tumor. Recent studies have shown that a distal clearance margin greater than 1.5 cm is sufficient when tumor histological differentiation is not poor to achieve curative resection of low rectal tumors^[1]. These have established the feasibility of ultra-low anterior resection for tumors at level as low as 3 cm from the dentate line.

For the ultra-low tumors, i.e., tumor with lower margin situated within 1-3 cm above the dentate line, the mandatory surgical procedure is still controversial. It is hardly practical to apply conventional ultra-low anterior resection with autosuture instrumentation to achieve an adequate section margin, because of the extreme difficulty in placing a stapler across the optimal distal rectal margin. While most surgeons insist in performing APR in these cases, intersphincteric resection (ISR) has drawn increasing attention as it provides anus preservation and more clear vision for resection.

Many retrospective studies have pointed out that there is a good local control after sphincter-saving resection for rectal cancer^[2-11]; however, the majority of studies did not carry more than the 5-year follow-up interval and they examined all rectal tumors, many of which can be removed by low/ultra-low anterior resection. This study assessed the oncological outcome of patients with very low rectal cancer by intersphincteric resection to determine whether abdominoperineal resection could be abolished.

MATERIALS AND METHODS

Patients

There were two distinct periods in the present study. During period I, from January 1995 to December 1998, eight consecutive patients having a non-fixed rectal adenocarcinoma (tumor stage T2) without distant metastasis

were enrolled. All tumors were smaller than 5 cm in maximal diameter and the lower margin was within 1-3 cm from the dentate line. Each patient underwent ISR. Post-operative follow-up interval was over 5 years. During period II, from January 1999 to December 2003, 10 patients with ultra-low rectal adenocarcinoma underwent ISR. Among those, six patients with T3-4 tumors received preoperative concurrent chemoradiotherapy (CCRT). The median follow-up time was 30 (range, 18-47) mo.

Methods

ISR was performed according to the methods previously described by Schiessel *et al.*⁴¹ This procedure was initiated by placing the patient in the Sim's position for the anal approach, mucosal incision, further exposure of the internal anal sphincter and intersphincteric space. After meticulous hemostasis was reached in the operation field, followed by closure of the rectal stump, and the patient was placed in lithotomy position to facilitate low anterior resection of the rectum with total mesorectal excision by laparotomy. A colonic J-pouch in all the patients as 6-8 cm in length was constructed from the distal descending colon and/or proximal sigmoid colon with uniform linear staplers. Mobilization of the splenic flexure colon, descending colon, sigmoid colon and ligation of the inferior mesenteric vein at the inferior margin of the pancreas are critical components of this procedure to enable the pouch to be easily drawn toward the anus. The constituted reservoir was then anastomosed to the dentate line with interrupted sutures. Under direct vision, the external anal sphincter was preserved to maintain defecation function.

During period II, ISR was performed on 10 patients with the same tumor location as period I. Among those, six patients with fixed tumors (T3-4) underwent preoperative CCRT (5 040 cGy in 28 fractions over 6 wk with continuous infusion of 5-FU and LV on the first and last 5 d during radiotherapy).

All patients in period I and period II were performed temporary diverting colostomy. Follow-up evaluations were performed on an outpatient basis. As to that whether tumor recurrence or distant metastasis existed, it was determined by digital rectal examination, clinical symptoms, measurement of serum tumor marker level and image study facilities.

RESULTS

Clinical and pathological data

There were eight patients enrolled in the period I from 1995 to 1998, 2 males and 6 females, median age was 61 years (range: 44-79 years). Pathologic staging as TNM categorization for these 8 patients comprised 7 T2N- and 1 T2N+. On the other hand, there were 10 patients recruited in the period II since 1999 till 2003, 5 males and 5 females, median age was 62 (range: 42-72) years. No statistic difference in age or gender between period I and period II was proved (Table 1).

Among the period II group, six patients with fixed tumors were undertaken by the protocol of preoperative CCRT (5 040 cGy in 28 fractions over 6 wk concurring

Table 1 Characteristics of cases

| | Period I | Period II |
|--------------------|-------------------|------------------------|
| | 1995-01/1998-12 | 1999-01/2003-12 |
| Number of patients | 8 | 10 |
| Male | 2 | 5 |
| Female | 6 | 5 |
| Age range (yr) | 44-79 (median 61) | 42-72 (median 62) |
| Follow-up (mo) | 60 | 18-47 (median 30) |
| Tumor | | |
| Non-fixed (T2) | 8 | 4 |
| Fixed (T3-4) | 0 | 6 (pre-operative CCRT) |

Table 2 Pathologic results

| Clinical stage | Period I | Period II | |
|----------------|----------|--------------|----------------|
| | (n = 8) | T2Nx (n = 4) | T3-4Nx (n = 6) |
| | | | CCRT |
| T0 | 0 | 0 | 2 |
| T1N0 | 0 | 0 | 1 |
| T2N0 | 7 | 3 | 2 |
| T2N1 | 1 | 1 | 0 |
| T3Nx | 0 | 0 | 1 (T3N1) |

with continuous infusion 5-FU and LV on the first and last 5 d during radiotherapy). In comparing the tumor staging between pre- and post-CCRT, there were seven patients with a significant tumor regression status, including two complete regressions. Pathologic TNM classifications for the 10 patients were 2 T0, 1 T1N-, 5 T2N-, 1 T2N+ and 1 T3N+ (Table 2).

Regardless of period I or period II, curative resection of malignancy with microscopically clear oncologic section margin was confirmed by postoperative pathologic diagnosis in all the patients. The median distal resection margin was 1.5 (range: 0.5-2) cm. There was no postoperative mortality. Postoperative morbidity was found as follows: wound infection in 4/18 (22.2%), prolonged Foley catheter indwelling in 3/18 (16.7%), and urinary tract infection in 2/18 (11.1%).

All patients underwent interval colostomy closure at a median of 7 (range, 3-14) mo, no colostomy closure-related complication was proposed.

Postoperative local recurrence and distant metastasis

In the period I group, there were two patients who developed local recurrence/metastasis after at least a 5-year follow-up; it was comprised of local recurrence with multiple liver metastases in one patient and multiple lung metastases in another patient. Local recurrence rate was 12.5% and metastasis rate 25.0%; the 5-year survival rate was 87.5% and disease-free survival rate 75.0%. In the period II group, there was no local recurrence or distant metastasis in these 10 patients after a median follow-up of 30 (range: 18-47) mo (Table 3).

Functional outcome

None of the patients in the period I group could be traced for functional evaluation, but we appraised bowel function in the period II group by patient themselves using self-

Table 3 Recurrence, metastasis and survival

| | Period I (n = 8) | Period II (n = 10) |
|----------------------------|---------------------------------------|-----------------------|
| Follow-up (mo) | >60 | 18-47 (median 30) |
| Local recurrence | 1/8 (12.5%), 12 mo after operation | - |
| Distant metastasis | 2/8 (25%) | |
| Liver | 1/8, 52 mo after operation | |
| Lung | 1/8, 25 mo after operation | |
| Survival rate | 7/8 (87.5%) | 100% |
| Disease-free survival rate | 6/8 (75.0%) | 100% |

confession method in one year later after the colostomy being closed. Eight patients (80%) experienced six or fewer bowel movements per day, urgency was reported in 5 patients (50%), and most of them could tolerate anal continence status well. Only two patients stated that they were incontinent to liquid stools and required pad use at night time (Table 4).

DISCUSSION

The distal section margin of 1-2 cm would be currently considered sufficient for ultra-low rectal cancer in most instances^[1,5,7,8]. Parks *et al*^[12] reported that long-term survival and local recurrence rate after ISR were similar to those after APR. Several specialized studies have investigated ISR for low rectal cancer, and their local recurrence rates ranged from 0% to 12%^[2-11]; however, some studies reported that the patients with the location of rectal cancer at 5 cm more proximal to anal verge and did not always carried more than a 5-year follow-up interval. In this study, the local recurrence rate was 12.5% for T2-staged ultra-low rectal tumor after at least a 5-year follow-up. However, the number of patients in this study is small because of the highly selected criteria, size of the tumors smaller than 5 cm and the lower margin of tumors within 1 to 3 cm from the dentate line.

The radial involvement of a tumor is another critical predictive factor for local recurrence after rectal cancer resection. In most instances, patients with T3-4 carcinomas of the low-third rectum require APR. Recently, by using a multimodal approach, intersphincteric resection was practical in patients with T3-4 carcinomas of the lower third of the rectum^[7,10,11,13-15].

Preoperative CCRT reducing tumor volume, causing tumor down-staging, and further facilitating surgical resection of malignancy have been proposed. Recent studies concerning preoperative chemoradiotherapy have demonstrated that it improves local control and cancer-related survival. Rullier *et al*^[10] reported only 2% (1/43) local recurrence rate if combining preoperative CCRT and successive ISR for ultra-low rectal cancer were attempted (median follow-up was 30 mo)^[10]. Luppi *et al*^[13] reported 94% local control rate for T3-4 rectal cancer using preoperative chemoradiotherapy^[13]. Saito *et al* reported similar results for local control and acceptable anal function in a series of 35 patients (median follow-up: 23 mo), these patients had T3-staged ultra-low rectal cancer and were

Table 4 Functional outcome of patients in period II

| | Period II (n = 10, %) |
|--------------------------|--------------------------|
| Stool frequency: ≤3 | 5/10 (50) |
| 4-6 | 3/10 (30) |
| 7-9 | 1/10 (10) |
| ≥10 | 1/10 (10) |
| Urgency | 5/10 (50) |
| Continence: Perfect | 3/10 (30) |
| Incontinence of flatus | 2/10 (20) |
| Minor soiling to liquids | 3/10 (30) |
| Major soiling to liquids | 2/10 (20) |
| Incontinence to solids | 0/10 (0) |

treated by preoperative CCRT with consecutive ISR^[7].

In the past 4 years, 10 patients with ultra-low rectal cancer were managed in this study by multimodality treatment and no distant metastasis was disclosed during the follow-up period. Preoperative CCRT was applied to six T3-4 staged patients, and this treatment enhanced tumor shrinkage more than 25% in five patients; it is inspiring that no residual cancer was identified in 2 patients among these 5 patients. After a median follow-up of 30 mo, there was no local recurrence or distant metastasis developed.

Cluster defecation associated with tenesmus, urgency, and incontinence are not uncommon in straight coloanal anastomosis. The colonic pouch could convert the functional deficiencies associated with the loss of rectal capacity and reduced compliance resulting from straight coloanal anastomosis. Lazorthes *et al*^[16] and Parc *et al*^[17] conducted a colonic J pouch anastomosing at the dentate line, and they assumed superior functional outcome compared to straight anastomosis^[16,17]. A number of clinical series have evidence to support that utilizing a colonic pouch anal anastomosis could enhance the functional results and postoperative quality of life of patients after rectal cancer resection^[16-19,21,22]. The functional advantages of a colonic pouch anal anastomosis have been achieved within a shorter period than straight coloanal anastomosis after surgery. This superiority in J pouch groups remains evident when compared with the straight coloanal anastomosis 2 years after the surgery as reported by Joo *et al*^[21] and Sailer *et al*^[22].

Optimal pouch size remains unclear. Numerous J pouch sizes have been attempted, ranged from 5 to 12 cm in length. Increasing pouch length is always associated with incomplete evacuation and requires suppositories or enemas to rescue such sufferings. Parc recommended an adequate limb length of 8-10 cm^[17]. Hida *et al*^[20] proposed that a 5-cm pouch could function as adequate reservoir, and it is technically easy with requiring not too long a segment of bowel for construction^[20]. Clinical experience to date favors that the optimal pouch is achieved by employing two 6-7 cm limbs of the colon. In this study, a 6-8 cm pouch was constructed and harvested proper functional outcome. During the time the colonic Jpouch was constituted, the distal colon must be mobilized and adequate perfusion should be preserved to prevent complications such as mobilized colon ischemic change or

anastomotic insufficiency. We believe that these standard surgical issues have facilitated the achievement of tolerable functional results.

In conclusion, ISR could achieve acceptable local control, cancer-related survival and avoid permanent stoma in patients with early staged ultra-low rectal cancer. Preoperative CCRT facilitates intersphincteric curative resection in patients with T3-4 staged ultra-low rectal cancer. Longer follow-up of these patients should be recommended, especially for those receiving preoperative chemoradiotherapy.

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Role of transforming growth factor-beta1-smad signal transduction pathway in patients with hepatocellular carcinoma

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Abstract

AIM: To explore the role of transforming growth factor-beta1 (TGF- β 1)-smad signal transduction pathway in patients with hepatocellular carcinoma.

METHODS: Thirty-six hepatocellular carcinoma specimens were obtained from Qidong Liver Cancer Institute and Department of Pathology of the Second Affiliated Hospital of Nanjing Medical University. All primary antibodies (polyclonal antibodies) to TGF- β 1, type II Transforming growth factor-beta receptor (T β R-II), nuclear factor-kappaB (NF- κ B), CD34, smad4 and smad7, secondary antibodies and immunohistochemical kit were purchased from Zhongshan Biotechnology Limited Company (Beijing, China). The expressions of TGF- β 1, T β R-II, NF- κ B, smad4 and smad7 proteins in 36 specimens of hepatocellular carcinoma (HCC) and its adjacent tissue were separately detected by immunohistochemistry to observe the relationship between TGF- β 1 and T β R-II, between NF- κ B and TGF- β 1, between smad4 and smad7 and between TGF- β 1 or T β R-II and microvessel density (MVD). MVD was determined by labelling the vessel endothelial cells with CD34.

RESULTS: The expression of TGF- β 1, smad7 and MVD was higher in HCC tissue than in adjacent HCC tissue ($P < 0.01$, $P < 0.05$, $P < 0.01$ respectively). The expression of T β R-II and smad4 was lower in HCC tissue than in

its adjacent tissue ($P < 0.01$, $P < 0.05$ respectively). The expression of TGF- β 1 protein and NF- κ B protein was consistent in HCC tissue. The expression of TGF- β 1 and MVD was also consistent in HCC tissue. The expression of T β R-II was negatively correlated with that of MVD in HCC tissue.

CONCLUSION: The expressions of TGF- β 1, T β R-II, NF- κ B, smad4 and smad7 in HCC tissue, which are major up and down stream factors of TGF- β 1-smad signal transduction pathway, are abnormal. These factors are closely related with MVD and may play an important role in HCC angiogenesis. The inhibitory action of TGF- β 1 is weakened in hepatic carcinoma cells because of abnormality of TGF- β 1 receptors (such as T β R-II) and postreceptors (such as smad4 and smad7). NF- κ B may cause activation and production of TGF- β 1.

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Key words: TGF- β 1; T β R-II; Smad4; Smad7; NF- κ B; MVD; Hepatocellular carcinoma; Signal transduction

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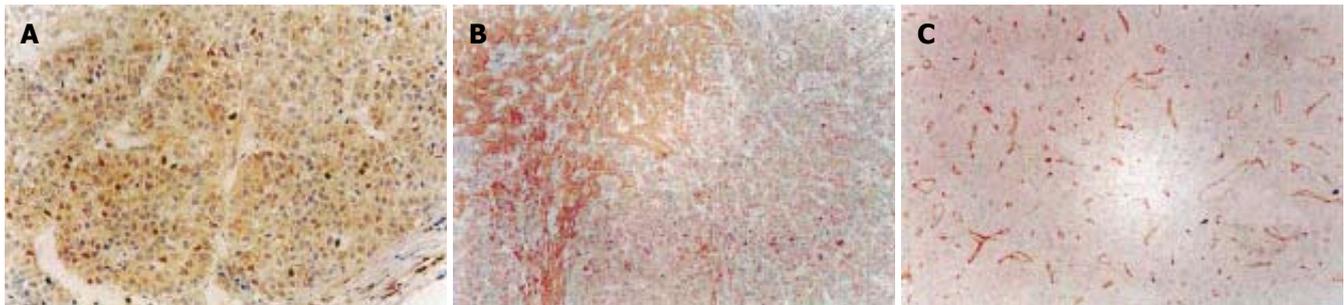
INTRODUCTION

Hepatocellular carcinoma is one of the most common malignant tumors in the world. Recent studies have demonstrated that the key factor of invasiveness and metastasis of HCC is neovascularization. There are two kinds of factors in adjusting neovascularization, one is positive adjusting factors, such as vascular endothelial cell growth factor (VEGF), hepatocellular growth factor (HGF), transforming growth factor alpha (TGF- α), epidermal growth factor (EGF). The other kind includes negative adjusting factors, such as transforming growth factor beta (TGF- β). Synthesis of TGF is adjusted by nucleus transcription factors, such as NF- κ B. This study was designed to investigate the significance and mechanism of TGF- β 1-smad signal transduction pathway in hepatocellular carcinoma (HCC).

Table 1 Expressions of TGF- β 1, T β R-II in HCC and its adjacent tissue (mean \pm SD)

| | n | TGF- β 1 | | n | T β R-II | |
|-----------------|----|----------------------------------|--------------------------------|----|----------------------------------|--------------------------------|
| | | Average optical density (OD) | Positive area (%) | | Average optical density (OD) | Positive area (%) |
| HCC tissue | 24 | 0.0704 \pm 0.0116 ^a | 63.08 \pm 12.91 ^b | 18 | 0.0406 \pm 0.0121 ^c | 31.33 \pm 16.35 ^d |
| Adjacent tissue | 24 | 0.0462 \pm 0.0110 | 24.04 \pm 16.68 | 18 | 0.0639 \pm 0.0129 | 58.33 \pm 11.48 |

$t=7.39$, ^a $P<0.01$ vs adjacent tissue; $t=9.24$, ^b $P<0.01$ vs adjacent tissue; $t=5.58$, ^c $P<0.01$ vs adjacent tissue; $t=5.74$, ^d $P<0.01$ vs adjacent tissue.

**Figure 1** Expression of TGF- β 1 (A), T β R-II (B) and MVD (C) in HCC and its adjacent tissue.

MATERIALS AND METHODS

Materials

Thirty-six HCC specimens were obtained from Qidong Liver Cancer Institute and Department of Pathology of the Second Affiliated Hospital of Nanjing Medical University. All primary antibodies (polyclonal antibodies) to TGF- β 1, T β R-II, NF- κ B, CD34, smad4 and smad7 and secondary antibodies were purchased from Zhongshan Biotechnology Limited Company (Beijing, China).

Methods

Each specimen was cut into 4- μ m thick sections. Tissue wax sections were unfolded on glass sheet. Immunohistochemistry (streptol-in-biotin-peroxidase method) was used to detect the expression of TGF- β 1, T β R-II, NF- κ B, CD34, smad4 and smad7. Briefly, paraffin-embedded tissue sections were dewaxed, treated with 3% H₂O₂ at 37 °C, washed with PBS, incubated with TGF- β 1, T β R-II, NF- κ B, CD34, smad4 and smad7 antibodies separately, washed with PBS for 15 min, incubated again with streptol-in-biotin-peroxidase at 37 °C. Finally, the sections were washed with PBS for 15 min, visualized with DAB reagent and counterstained with hematoxylin. Negative and positive controls were used simultaneously to ensure specificity and reliability of the staining process. The negative controls were incubated with PBS instead of primary antibody and a positive section supplied by the manufacturer of the staining kit was taken as positive control. Sections were observed under microscope after being mounted. All positive sections were analyzed with RY2000 analysis system. Microvessel density (MVD) was measured as previously described [1]. High vessel density was found in 100 \times sights. Microvessels in five regions were counted in 400 \times sights, the average of microvessels with CD34 staining in

five regions was calculated as MVD.

Result judgement

The buffy staining of cell membrane, plasma or nuclei was considered positive staining of TGF- β 1, T β R-II, NF- κ B, smad4 and smad7 (Figures 1A and 1B). Positive staining of CD34 was considered as microvessels (Figure 1C).

Statistical analysis

The data were expressed as mean \pm SD. Chi-square test and t test were used. $P<0.05$ was considered statistically significant. Kappa value was regarded as consistency degree.

RESULTS

Expressions of TGF- β 1 and T β R-II in HCC and its adjacent tissue

Different expressions of TGF- β 1, T β R-II were observed in HCC and its adjacent tissue. There was a significant difference in the positive expressions of TGF- β 1, T β R-II between HCC and its adjacent tissue (Table 1).

Relationship between expressions TGF- β 1 and T β R-II in HCC tissue

The positive expression rate of both TGF- β 1 and T β R-II was 27.78% (10/36) and their negative expression rate was 11.11% (4/36). The total rate was 38.89% (14/36). The discrepancy between expressions of TGF- β 1 and T β R-II was 61.11% (22/36). The consistency degree in statistical test was weak (kappa = 0.25) (Table 2).

Comparison of MVD in HCC and its adjacent tissue

The MVD was higher in HCC tissue than in its adjacent tissue. There was a significant difference in MVD between

Table 2 Relationship between expressions of TGF- β 1 and T β R-II in HCC tissue

| | | TGF- β 1 | | Total |
|-----------------|---|----------------|----|-------|
| | | + | - | |
| T β R- II | + | 10 | 8 | 18 |
| | - | 14 | 4 | 18 |
| Total | | 24 | 12 | 36 |

HCC and its adjacent tissue ($P < 0.01$, Table 3).

Relationship between MVD in HCC tissue and expression of TGF- β 1 or T β R- II

The MVD in HCC tissue was higher in positive expression group of TGF- β 1 than in negative expression group of TGF- β 1. The difference was significant between the two groups ($P < 0.05$). The MVD in HCC tissue in positive expression group of T β R- II was lower than in negative expression group of T β R- II. The difference was significant between the two groups ($P < 0.01$).

Relationship between expression of TGF- β 1 and NF- κ B in HCC tissue

Among the 36 specimens, 19 (52.78%) had positive expression of TGF- β 1 and NF- κ B in HCC tissue. The negative expression rate of TGF- β 1 and NF- κ B was 22.22% (8/36) in HCC tissue. The consistent rate of TGF- β 1 and NF- κ B was 75.00% (27/36).

Expressions of smad4 and smad7 in HCC and its adjacent tissue

The positive expression rate of smad4 in HCC and its adjacent tissue was 19.44% (7/36) and 75.00% (21/28) respectively. There was a significant difference between them ($P < 0.01$). The positive expression rate of smad7 in HCC tissue and its adjacent tissue was 63.89% (23/36) and 35.71% (10/28) respectively. There was a significant difference between them ($P < 0.05$).

DISCUSSION

Tumor growth, invasion, metastasis depend on angiogenesis. Through its vessels, tumor can obtain rich nutrients and secrete tumor cells, resulting in tumor growth and metastasis. MVD of tumor is a valid marker to reflect tumor angiogenesis. Chen *et al*^[2] reported that HCC MVD can be reflected by dynamic enhancement of spiral CT scanning. MVD of tumor has been detected by marking vessel endothelium. Vascular endothelial growth factor (VEGF) is an important angiogenic factor regulating tumor angiogenesis. Yao *et al*^[3] reported that the VEGF expression rate is 63.9% in HCC, 78.3% in non-encapsulated HCC, and 90.9% in HCC with extrahepatic metastasis, respectively. The VEGF expression is closely correlated with MVD. Moon *et al*^[4] reported that overproduction of VEGF and angiopoietin 2 in HCC cells may increase vascularity and tumor growth in a paracrine manner. Poon *et al*^[5] reported that the MVD in HCC tissue is higher than that in its adjacent tissue. The MVD in

Table 3 Comparison of MVD in HCC and its adjacent tissue (mean \pm SD)

| | <i>n</i> | MVD |
|-----------------|----------|-------------------|
| HCC tissue | 36 | 32.45 \pm 10.62 |
| Adjacent tissue | 36 | 4.62 \pm 1.67 |

HCC tissue is associated with hepatocarcinoma prognosis. The higher the MVD is, the poorer the prognosis is. Our study revealed the same result, suggesting that tumor angiogenesis plays an important role in tumor genesis and development.

TGF- β is an important adjusting factor and has five isoforms: TGF- β 1-5. TGF- β 1-3 have the same biological functions with 70-80% homology. TGF- β 1 is a negative adjusting factor in tumor growth. There are three kinds of TGF- β receptors: T β R-I, T β R-II, T β R-III. Their relative molecules are 65×10^3 , $85-110 \times 10^3$ and 6×10^5 respectively. T β R-I and T β R-II playing a part in signal transduction are serine/ threonine protein kinase receptors with a single span membrane. The section of extracellular membrane is short, while the section of cell plasma is long. The section of extracellular membrane contains rich cysteine, the section of cell plasma contains serine/ threonine protein kinase structure and possess its activity. Compared to T β R-II, the out and inner sections of cellular membrane of T β R-I are short. TGF- β 1 combines with T β R-II becoming dimer, then with T β R-I becoming trimer. The activated T β R-I and phosphate-acidulated smad could adjust transcription of genes. TGF- β 1 is over-expressed in tumor tissue, but tumor cells do not respond to suppressive factor TGF- β 1, suggesting that the signal transduction pathway is abnormal. TGF- β plays an important role as a ligand in tumor genesis suppressing cell growth in early stage and promoting cell growth in advanced stage. TGF- β 1 exerts its strong suppressive effect on hepatocellular proliferation. Kim *et al*^[6] showed that TGF- β 1 could induce hepatocyte apoptosis. Abou-Shady *et al*^[7] detected TGF- β mRNA in normal and HCC tissue with Northern blot method and found that TGF- β mRNA is over-expressed in HCC and its adjacent tissue. Idobe *et al*^[8] compared the intensity of TGF- β 1 staining in HCC and its adjacent tissue and found that the former is more intense than the latter. The expression of TGF- β 1 in HCC tissue is correlated with the histological differentiation, namely the lower the histological differentiation of HCC, the more intense the TGF- β 1 staining in HCC tissue. Matsuzaki *et al*^[9] showed that TGF- β 1 mRNA is over-expressed in HCC cells and TGF- β 1 accelerates their proliferation. Giannelli *et al*^[10] reported that invasive HCC cells express alpha3 beta1-integrin whereas noninvasive HCC cells do not. TGF- β 1 stimulates alpha3-integrin expression at a transcriptional level in noninvasive HCC cells, causing transformation into a motile and invasive phenotype. Invasive HCC cells secrete abundant active TGF- β 1 in comparison to noninvasive HCC cells. Anti-TGF- β 1 neutralizing antibody reduces alpha3-integrin expression and the ability of HCC to invade

cells, suggesting that TGF- β 1 may play an important role in HCC invasiveness by stimulating α 3-integrin expression, and may be an important target for new therapies. Cai *et al*^[11] showed that TGF- β 1 treatment can enhance the amount of α 5 β 1-integrin on HCC cell surface, the mRNA level of α 5 subunit, subsequently stimulating cell adhesion to fibronectin (Fn) and laminin (Ln) matrix. TGF- β 1 can also promote cell migration. Song *et al*^[12] reported that TGF- β 1 may be a useful serologic marker in detecting HCC at its initial stage because it shows higher sensitivity and specificity in the diagnosis of small HCC. Ueno *et al*^[13] reported that expression of T β R-II in liver tissues is significantly decreased in patients with HCC compared to that in patients with chronic hepatitis or liver cirrhosis. They transfected T beta RII cDNA to hepatoma cell line (Huh7) and compared the change of cell number and observed the induction of apoptosis after TGF-beta1 treatment using a FACScan flow cytometer. In Huh7 cells transfected with T beta RII cDNA, cell arrest and apoptosis were obviously induced. We have previously shown that expression of TGF- β 1 mRNA and protein in HCC tissue is higher than that in its adjacent tissue^[14]. Our study displayed that expression of TGF- β 1 was enhanced, and expression of T β R-II was weak in HCC tissue compared to that in its adjacent tissue, which may be due to the lower expression of T β R-II in HCC cells^[13] that can escape from the inhibitory effects of TGF- β 1, thus causing genesis and development of HCC.

Smad is an essential signal transducer of TGF-beta signal pathway. At present, ten kinds of smad have been found: smad1-10. Smad4 is an action substrate of TGF- β receptor and has been identified as a tumor suppressor gene. Mutation or lower expression of smad4 has been observed in many kinds of tumor^[15-18]. In normal hepatic tissue, smad4 plays an essential role in signal transduction of TGF- β and influences gene transcription, controls cell growth and guides the suppressive role of TGF- β in hepatic cell growth. Tannapfel *et al*^[19] reported that expression of smad4 is decreased in HCC tissue. Our study showed that expression of smad4 was lower in HCC tissue than in its adjacent tissue, suggesting that expression of smad4 is abnormal in HCC tissue, resulting in blocking signal transduction of TGF- β 1 and taking part in hepatic carcinoma cell escaping from the suppression of TGF- β 1. smad7 is an inhibitor of smad and can bind to T β R-I, blocking phosphorylation of smad and signal transduction of TGF- β 1. It is thus considered as an oncogene. Kawate *et al*^[20] investigated mutation of smad7 in HCC tissue using polymerase chain reaction-single strand conformation polymorphism analysis and found that smad7 displays single nucleotide polymorphisms. Our study showed that expression of smad7 was higher in HCC tissue than in its adjacent tissue, suggesting that smad7 may take part in hepatic carcinoma cell escaping from suppression of TGF- β 1.

Synthesis of TGF- β 1 is controlled by nuclear transcription factors. At present, the main nuclear transcription factors are nuclear factor-kappaB (NF- κ B) and activator protein 1. NF- κ B could bind to enhancer κ B of immunoglobulin kappa light chain gene. NF- κ B is a dimer of p65 and p50. In normal conditions, NF- κ B exists

in cell plasma. Inhibitory factor of NF- κ B (I- κ B) falls off NF- κ B complex after stimulated. Activated NF- κ B enters into nuclei and accelerates transcription of target gene. Because tissue transglutaminase gene promoter (a TGF- β 1 activated factor) contains binding sites of NF- κ B, NF- κ B can accelerate expression of TGF- β 1. It was reported that hepatic carcinoma cells could excrete TGF- β 1^[21]. Our study showed that expression of NF- κ B was closely related with TGF- β 1 expression.

In conclusion, TGF- β 1, T β R-II, NF- κ B, smad4 and smad7 in HCC tissue are the major factors in TGF- β 1-smad signal transduction pathway. These factors are closely related with MVD and may play an important role in HCC angiogenesis. NF- κ B may cause the activation and production of TGF- β 1.

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Acute duodenal Crohn's disease successfully managed with low-speed elemental diet infusion *via* nasogastric tube: A case report

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Abstract

Duodenal Crohn's disease is rare, and patients without obstruction are treated medically. We herein report one case whose duodenal Crohn's disease was successfully managed with low-speed elemental diet infusion through a nasogastric tube. A 28-year-old female developed acute duodenal Crohn's disease. Upper GI radiologic and endoscopic examinations showed a stricture in the duodenal bulb. Using the duodenal biopsy specimens, mucosal cytokine levels were measured; interleukin (IL)-1 β , IL-6, IL-8, and tumor necrosis factor- α levels were remarkably elevated. For initial 2 wk, powdered mesalazine was orally given but it was not effective. For the next 2 wk, she was treated with low-speed elemental diet therapy using a commercially available Elental™, which was infused continuously through a nasogastric tube using an infusion pump. The tip of the nasogastric tube was placed at an immediate oral side of the pylorus. The infusion speed was 10 mL/h (usual speed, 100 mL/h). After the 2-wk treatment, her symptoms were very much improved, and endoscopically, the duodenal stricture and inflammation improved. The duodenal mucosal cytokine levels remarkably decreased compared with those before the treatment. Although our experience was limited, low-speed elemental diet infusion through a nasogastric tube may be a useful treatment for acute duodenal Crohn's disease.

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Key words: Cytokine; Duodenal Crohn's disease; Elemental diet; Inflammation; Stricture

Yamamoto T, Nakahigashi M, Umegae S, Kitagawa T, Matsumoto K. Acute duodenal Crohn's disease successfully managed with low-speed elemental diet infusion *via* nasogastric

INTRODUCTION

The frequency of duodenal Crohn's disease is rare, and is reported to range between 0.5% and 5.0% in Crohn's disease^[1-3]. Duodenal Crohn's disease is almost invariably present in association with the disease elsewhere in the intestinal tract. Medical therapy for distal disease may mask the symptoms of duodenal disease and delay its diagnosis. The most common site of duodenal Crohn's disease is the duodenal bulb, and stricture is the most common pathology^[1-3]. Medical therapy including H₂-receptor antagonists, 5-aminosalicylic acid, corticosteroids, and immunosuppressive drugs is effective for patients having duodenal strictures without obstruction; however, surgery is necessary for patients with obstruction^[1-3].

Cytokines are involved in the modulation of the immune system related to the pathogenesis of inflammatory bowel disease, such as Crohn's disease and ulcerative colitis, and they are rapidly synthesized and secreted by inflammatory cells^[4,5]. In our previous study, we found that elemental diet therapy markedly improved clinical symptoms, and reduced the mucosal cytokine production in patients with ileal or colonic Crohn's disease^[6]. Recently, we experienced one case whose duodenal Crohn's disease was successfully managed with low-speed elemental diet infusion through a nasogastric tube.

CASE REPORT

In June 2000, a 23-year-old female presented with abdominal pain and diarrhea, and she was diagnosed as having Crohn's disease in the terminal ileum. Thereafter, she was treated with corticosteroid (prednisolone, 5-20 mg/d) and mesalazine (Pentasa, 3.0 g/d). Despite the medical treatment, she required an operation (terminal ileal resection) in April 2002. After the initial operation, she was taking mesalazine (Pentasa, 3.0 g/d) to prevent recurrence. She started an enteral nutritional therapy using elemental diet, but could not continue the treatment. She developed abdominal pain and diarrhea due to recurrent ileal Crohn's disease, but

Table 1 Mucosal cytokine production in the duodenal bulb before and after elemental diet therapy

| Tissue cytokine (ng/g) | Before treatment | After treatment | Normal control ¹ median value (range) |
|------------------------|------------------|-----------------|--|
| IL-1 β | 370 | 45 | 12 (0-23) |
| IL-6 | 12 000 | 570 | 220 (70-280) |
| IL-8 | 450 | 30 | 20 (0-36) |
| TNF- α | 310 | 24 | 14 (0-21) |

¹Five patients without any gastroduodenal diseases.



Figure 1 Upper GI barium study at admission. A stricture was observed in the duodenal bulb.

she refused to receive any steroids or immunosuppressive drugs. In June 2003, she underwent reoperation (ileal resection). Thereafter, she was treated with mesalazine (Pentasa, 3.0 g/d).

In March 2005, she developed severe postprandial upper abdominal pain, nausea, and vomiting. Upper GI barium study showed a stricture of the duodenal bulb (Figure 1). Endoscopic examination revealed the stricture with mucosal edema and contact bleeding in the duodenal bulb (Figure 2A). The endoscope could be inserted into the second part of the duodenum, where the longitudinal ulcerations were observed (Figure 2B). The gastric mucosa was moderately inflamed but there were no ulcerations in the stomach. Histologically, the biopsy specimens of the duodenal bulb showed severe inflammation with the accumulation of a large amount of inflammatory cells. Granulomas were not detected. Using the duodenal biopsy specimens, mucosal cytokine levels were measured by the enzyme-linked immunosorbent assay as previously reported^[6]. The cytokine measurement was performed after the clinical data has been recorded. Laboratory investigators were blind to the clinical data. Interleukin (IL)-1 β , IL-6, IL-8, and tumor necrosis factor (TNF)- α levels were remarkably elevated compared with those in patients without any gastroduodenal diseases (normal control) (Table 1). She was diagnosed with acute duodenal Crohn's disease, and was hospitalized in our unit. At admission, the main results of blood examinations were: white blood cell count, 5.6×10^9 (normal, 3.5×10^9 - 9×10^9)/L; hemoglobin, 99 (normal, 115-160) g/L; platelet count, 542×10^9 (normal, 130×10^9 - 370×10^9)/L; erythrocyte sedimentation rate, 9 (normal, ≤ 15) mm/h; C-reactive protein, 4.5 (normal, ≤ 4) mg/L;

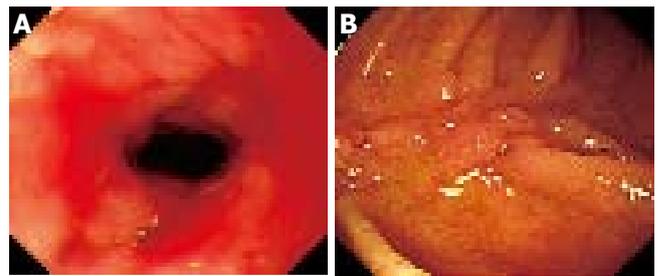


Figure 2 Endoscopic examination at admission. **A:** A stricture with mucosal edema and contact bleeding in the duodenal bulb; **B:** longitudinal ulcerations in the second part of the duodenum.



Figure 3 Endoscopic examination after the elemental diet therapy. Improvement of the duodenal stricture and inflammation was observed.

and albumin, 29 (normal, ≥ 40) g/L. Because she could not take any foods and her nutritional status was poor, she was managed with total parenteral nutrition (approximately 8368 kJ/d) after the admission. H₂-receptor antagonist (famotidine; Gaster, 40 mg/d) was intravenously administered for 4 wk. For initial 2 wk after the admission, powdered mesalazine (Pentasa, 1 g) was orally given three times a day. However, her symptoms were not improved, and repeat endoscopic examination also revealed no improvement. For the next 2 wk, she was treated with low-speed elemental diet infusion therapy. The enteral formula was a commercially available ElentalTM (Ajinomoto and Morishita-Russel, Tokyo, Japan), which is composed of amino acids, very little fat, vitamins, trace elements and a major energy source, dextrin. The details of ElentalTM were reported in our previous paper^[6]. The elemental formula was infused continuously through a silicone-elastomer nasogastric tube using an infusion pump. The tip of the nasogastric tube was placed at an immediate oral side of the pylorus, which was confirmed radiologically. The infusion speed was 10 mL/h (usual speed for this enteral formula is approximately 100 mL/h). After the 2-wk treatment, her symptoms were very much improved, and endoscopic examination also revealed improvement of the duodenal stricture and inflammation (Figure 3). The longitudinal ulcerations in the second part of the duodenum also improved but they did not normalize. The duodenal mucosal IL-1 β , IL-6, IL-8, and TNF- α levels also remarkably decreased compared with those before

the elemental diet therapy (Table 1). At present, she is taking low residue and low fat foods in the daytime according to the instructions of her dietician, and in the nighttime, the elemental diet is infused through a self-intubated nasogastric tube using an infusion pump with an increased speed (100 mL/h).

DISCUSSION

In this study, our patient refused to receive any steroids or immunosuppressive drugs in the management of Crohn's disease. Initially, powdered mesalazine (Pentasa, 3 g/d) was not effective for her duodenal Crohn's disease. We, therefore, decided to try our elemental diet therapy because there seemed to be no other useful medical treatment. The tip of the nasogastric tube was carefully placed at an immediate oral side of the pylorus. The elemental formula was infused continuously with a low speed (10 mL/h) for 2 wk because the duodenal bulb was obstructive. During the treatment, our patient did not have increased abdominal pain, nausea or vomiting. The aim of this therapy was to reduce the mucosal inflammation using the elemental diet, but not to improve the nutritional status of the patient. The enteral formula infused through the nasogastric tube continuously stayed at the site of the duodenal stricture. Eventually, this treatment remarkably reduced proinflammatory cytokine production in the duodenal mucosa. Clinical and endoscopic improvements were associated with a decline in the mucosal cytokine production. However, from our experience, the elemental diet is useful only for bowel strictures due to acute mucosal inflammation (edema). Generally, the tight fibrotic strictures require surgical treatment in patients with Crohn's disease.

In our previous prospective study^[6], 28 patients with active ileal or colonic Crohn's disease were treated with ElentalTM for 4 wk. The mucosal biopsies were obtained from the terminal ileum and large bowel before and after the treatment. After the treatment, clinical remission was achieved in 71% of patients. Endoscopic healing and improvement rates were 44% and 76% in the terminal ileum and 39% and 78% in the large bowel, respectively. The mucosal concentrations of IL-1 β , IL-6, IL-8, and TNF- α in the ileum and large bowel were increased before the treatment; however, these cytokine levels decreased to the levels of normal control after the treatment. Other studies also examined the relationship between the mucosal cytokine production and enteral nutrition using different formulas, and they reported similar results to that of ours^[7,8]. Breese *et al*^[7] investigated the proportion of lymphokine

(IL-2 and interferon- γ)-secreting cells in the intestinal mucosa of patients with Crohn's disease treated with either enteral nutrition or cyclosporine therapy. Enteral nutrition produced a reduction in lymphokine-secreting cells equivalent to cyclosporine therapy, and it produced better clinical improvement. Fell *et al*^[8] examined 29 children with active Crohn's disease treated with an oral polymeric diet. Macroscopic mucosal healing in the terminal ileum and colon was associated with a decline in ileal and colonic IL-1 β levels. The detailed mechanism of elemental diet on immunological reactions in Crohn's disease remains unknown. The main mechanisms may be removal of food antigen and decreases of bowel secretion and motility induced by very low fat content. However, further investigations are necessary.

Our low-speed elemental diet infusion therapy through a nasogastric tube was useful to improve acute duodenal Crohn's disease without any adverse effects. Although our experience was limited, this therapy may be a preferable treatment for acute duodenal Crohn's disease because patients can be treated without steroids or immunosuppressive drugs.

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CASE REPORT

Hepatic adenomatosis associated with hormone replacement therapy and hemosiderosis: A case report

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Abstract

We have reported a case of hepatic adenomatosis associated with hormone replacement therapy (estrogen and progesterone) and hemosiderosis caused by excessive blood transfusion for the treatment of chronic myeloid leukemia. A 34-year-old woman was found to have several hepatic tumors on a routine medical examination. The general condition was good. Laboratory studies showed iron overload. Abdominal computed tomography and selective hepatic angiography showed several hypervascular tumors in the right lobe of the liver (up to 20 mm in diameter). Since hepatocellular carcinoma could not be ruled out, subsegmental hepatectomy was performed. Histopathological examination of the surgical specimen showed hepatic adenomatosis with hemosiderosis. Both hormone replacement therapy and iron overload could be the cause of hepatic adenomatosis.

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Key words: Hepatic adenomatosis; Estrogen; Iron overload

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INTRODUCTION

Hepatic adenoma is an uncommon benign liver tumor; however, its incidence increases in women of childbearing age because of its close relation to the usage of oral contraceptives^[1]. Most patients start to develop hepatic adenoma after 5 years of usage of oral contraceptives^[2]. Hepatic adenoma frequently regresses following discontinuation of oral contraceptives^[3]. Hepatic adenoma also occurs in association with androgenic steroid use and with type I glycogen storage disease^[4]; however, the mechanism of its occurrence is unknown. On the other hand, hepatic adenomatosis is characterized by the presence of multiple adenomas of various sizes in the liver and it has neither female predominance nor relation with estrogen/progesterone intake^[5]. Thus, hepatic adenomatosis can be distinguished from hepatic adenoma. Both hepatic adenoma and adenomatosis are frequently difficult to distinguish from well-differentiated hepatocellular carcinoma (HCC). It has been reported that iron overload in the liver is occasionally associated with the development of both HCC and adenoma^[6]. HCC develops in a variety of conditions associated with iron overload, particularly idiopathic hemochromatosis in which a 30% incidence has been reported^[6]. Two cases of hepatic adenoma have been reported in patients with beta-thalassemia and secondary iron overload^[7,8]. Furthermore, one case of hepatic adenoma has been reported in a patient with aplastic anemia treated with anabolic androgenic steroids and blood transfusions for 5 years^[9]. Hepatic adenomatosis is also associated with hereditary hemochromatosis^[10]. Based on these findings, iron overload seems to be involved in the hepatocarcinogenic process. While the mechanism of hepatic tumorigenesis in the presence of hemosiderosis is not understood, it is possible that the Fenton's reaction generates free oxygen radicals that in turn cause DNA damage^[11].

Here we have reported a case of hepatic adenomatosis complicated with hepatic hemosiderosis caused by excessive blood transfusion for the treatment of chronic myeloid leukemia (CML), followed by sex steroid hormone therapy for 8 years.

CASE REPORT

A 34-year-old woman was found to have four hepatic tumors by computed tomography (CT) on a routine medical check-up. At 22 years of age, she was diagnosed

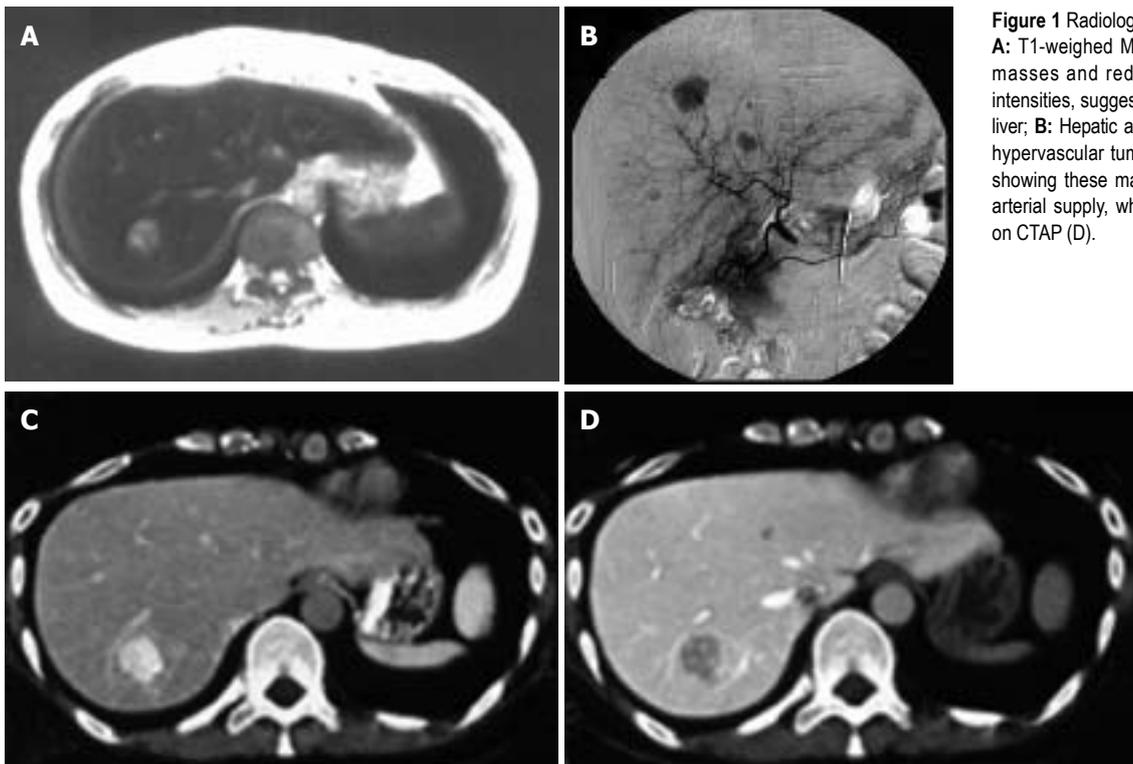


Figure 1 Radiological features of liver tumor. **A:** T1-weighted MRI showing high-intensity masses and reduced liver parenchymal intensities, suggestive of iron deposition in the liver; **B:** Hepatic arteriogram showing several hypervascular tumors in the liver; **C:** CTHA showing these mass lesions with increased arterial supply, which reduced portal supply on CTAP (**D**).

with CML. One year later, blast crisis occurred and she underwent allogeneic bone marrow transplantation (BMT) from her daughter in addition to receiving total body irradiation (TBI) and several blood transfusions. She developed amenorrhea after TBI and started to receive hormone replacement therapy (estrogen and progesterone) from 26 years of age to maintain uterine and ovarian functions. She remained relatively healthy without recurrence of CML after BMT. However, liver tumors were found on regular examination 8 years after BMT.

She had no history of glycogen storage disease or usage of anabolic steroid. General physical examination was normal. Blood tests and urinalysis were normal except for elevated serum iron (2810 µg/L and ferritin (2508.2 µg/L). Hepatitis B surface antigen (HBsAg), anti-HBs antibody, and anti-hepatitis C virus antibody were negative. Serum alpha-fetoproteins (AFP), protein induced by the absence of vitamin K (PIVKA)-II, carcinoembryonic antigen (CEA), and carbohydrate antigenic determinant (CA) 19-9 were within normal limits.

Abdominal ultrasonography (US) demonstrated an iso-echoic mass lesion measuring 2 cm in diameter located in the right posterior segment of the liver. Although US showed only one tumor, abdominal CT showed several low-density lesions (up to 2 cm in diameter) in the liver (segments 4, 5, 7, and 8 according to Couinaud's classification). Dynamic CT revealed enhancement of the masses during arterial phase, which showed low-density lesions during portal venous phase. These abnormal masses in the liver were also detected as high-intensity on T1-weighted and iso-intensity on T2-weighted magnetic resonance imaging (MRI). Moreover, both T1-weighted and T2-weighted MRI showed decreased liver parenchymal intensities, suggestive of hemosiderosis (Figure 1A). Celiac

angiography demonstrated multiple hypervascular mass lesions in the liver (segments 4, 5, 7, and 8) (Figure 1B). However, neither arteriovenous shunts nor vessel invasion were observed. CT during hepatic angiography (CTHA) showed these mass lesions with increased arterial supply, which reduced portal supply on CT during arterial portography (CTAP) (Figures 1C and 1D).

A tissue specimen of the tumor obtained by fine needle biopsy revealed monotonous growth of tumor cells, but no atypia was observed. Based on the above findings, the preoperative diagnosis was hepatic adenomatosis, although HCC could not be completely ruled out. Therefore, subsegmental hepatectomy for segment 7 was performed. The tumor was yellowish and well-defined without encapsulation. Microscopically, the tumor cells were mostly normal in size. Their nuclei were round and regular in size, and their cytoplasm was eosinophilic and partly clear. The tumor cells were arranged predominantly in a thin trabecular pattern without pseudo-glandular structures and no portal triad was observed in the tumor (Figure 2A). The non-tumorous part of the liver showed marked hepatocellular and reticuloendothelial hemosiderosis (Figure 2B). Several small nodules could also be seen in the normal parenchyma around the tumor. The pathological diagnosis was hepatic adenomatosis in hemosiderosis of the liver. The patient was advised to stop hormonal therapy and remained healthy during the follow-up. At 3 years after discontinuation of hormonal therapy, the residual tumors did not change in size as shown by CT.

DISCUSSION

We have described here a woman with hepatic adenomatosis who had not used oral contraceptives

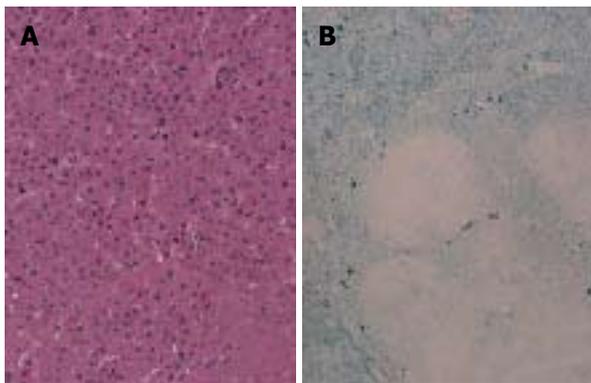


Figure 2 Macroscopic and microscopic examinations of tumor specimen. **A:** Macroscopic examination showing well-defined non-encapsulated tumor. Microscopically, the tumor cells are arranged predominantly in a thin trabecular pattern. No portal triad can be seen in the tumor (HE×200); **B:** The non-neoplastic hepatocytes are almost normal, but contain cytoplasmic hemosiderin (Berlin Blue×20). On the other hand, no iron deposition is seen in the adenoma nodule.

but received estrogen and progesterone for 8 years as hormone replacement therapy for early menopause caused by TBI. Baum *et al*^[2] have reported that the incidence of hepatic adenoma has increased among women on the usage of oral contraceptives^[1]. For example, about 90% of patients with hepatic adenoma had continuously taken oral contraceptives in the USA^[12]. In contrast, hepatic adenoma is rare in Japan because of the lack of widespread use of oral contraceptives; the incidence of hepatic adenoma in Japanese women on oral contraceptives is about 5%^[13]. On the other hand, hepatic adenomatosis is an extremely rare syndrome and defined as the presence of multiple (at least 10) adenomas in the liver. Flejou *et al*^[5] have indicated that liver adenomatosis affects both men and women, and it is not related to the usage of oral contraceptives, usage of androgenic steroid and glycogen storage disease. Our case had not used oral contraceptives but had received estrogen and progesterone.

Differential diagnosis of hepatic adenomatosis from multiple hemangiomas, multifocal nodular hyperplasia, multifocal HCC, and hypervascular liver metastases is sometimes difficult^[9]. In general, HCC shows a rapid progression resulting in the tumorous obstruction of the intrahepatic portal veins, and it is associated with increased serum AFP concentration and liver cirrhosis^[1]. On the other hand, hepatic adenomatosis shows a slow progression^[9], although it sometimes causes impaired liver function, hemorrhage, rupture, and malignant degeneration of the lesion^[8,14]. In our case, it was difficult to make differential diagnosis of hepatic adenomatosis from HCC based on the clinical, radiological, and histological examinations. Therefore, subsegmental hepatectomy was performed. Histopathological examination of a large specimen showed non-encapsulated nodule of tumor cells arranged predominantly in a thin trabecular pattern without portal vein or bile ducts. Several small nodules were also seen in the normal parenchyma around the tumor. These findings were compatible with hepatic adenomatosis. The extratumorous parenchyma showed hemosiderosis in hepatocytes; however, no iron deposition

was noted in the adenoma nodules.

The pathogenesis of hepatic adenomatosis remains unknown. Hepatocellular adenomas in beta-thalassemic patients with secondary iron overload have been reported^[7,8]. The development of HCC is associated with a variety of iron overload states, particularly hemochromatosis^[7]. Recently, a case with hepatic adenomatosis related to primary hemochromatosis was reported^[10]. Based on these findings, iron overload might be involved in the development of hepatocarcinogenesis. Moreover, Kebapci *et al*^[9] reported a patient with hepatic adenomatosis who developed blood transfusion-related hemosiderosis and had received anabolic steroids for the treatment of aplastic anemia. In this regard, elevated body iron stores is reported as a risk factor for the appearance of several tumors in human beings^[15]. Furthermore, it is considered that estrogen stimulates iron uptake and metabolism *in vivo* and the interaction of estrogen metabolites and metal ions produces free radical damage^[11,15]. Therefore, the relation between estrogen administration and hepatic hemosiderosis induced by secondary iron overload is likely involved in the development of hepatic adenomatosis in our case.

To the best of our knowledge, this is the first report of hepatic adenomatosis associated with hormone replacement therapy and secondary hemosiderosis. Thus, our case represents a human prototype of liver tumor formation induced by excessive iron and administration of sex steroid. Iron overload and sex hormone therapy may act synergistically to enhance the development of liver adenomatosis.

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CASE REPORT

Successful treatment of severe pouchitis with rebamipide refractory to antibiotics and corticosteroids: A case report

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Abstract

The antibiotics, metronidazole and ciprofloxacin, are the first-line treatment for pouchitis. Patients who do not respond to antibiotics or conventional medications represent a major challenge to therapy. In this report, we have described a successful treatment of severe refractory pouchitis with a novel agent, rebamipide, known to promote epithelial cell regeneration and angiogenesis. A 27-year-old male with ileo-anal pouch surgery presented with worsening anal pain, diarrhea, and abdominal pain. The patient was diagnosed to have pouchitis and was given metronidazole together with betamethasone enema (3.95 mg/dose). However, despite this intensive therapy, the patient did not improve. On endoscopy, ulceration and inflammation were seen in the ileal pouch together with contact bleeding and mucous discharge. The patient was treated with rebamipide enema (150 mg/dose) twice a day for 8 wk without additional drug therapy. Two weeks after the rebamipide therapy, stool frequency started to decrease and fecal hemoglobin became negative at the 4th wk. At the end of the therapy, endoscopy revealed that ulcers in the ileal pouch had healed with no obvious inflammation. The effect of rebamipide enema was dramatic and was maintained throughout the 11-mo follow-up. The patient continued to be in remission. No adverse effects were observed during the treatment or the follow-up period. The sustained response seen in this case with severe and refractory pouchitis indicates that agents, which promote epithelial cell growth, angiogenesis and mucosal tissue regeneration, are potential therapeutic agents for the treatment of refractory colorectal lesions.

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Key words: Refractory pouchitis; Rebamipide enema; Ileo-anal pouch; Epithelial cells

Miyata M, Konagaya T, Kakumu S, Mori T. Successful

INTRODUCTION

Ulcerative colitis is a debilitating inflammatory bowel disease that poorly responds to the current medical interventions with aminosaliclates, corticosteroids, immunomodulators, and more recently with novel biologicals. Patients who fail to respond to the mainstays of therapy until now have had limited options, but submit to colectomy^[1]. Accordingly, proctocolectomy and ileo-anal pouch surgery (IPAA) have become the standard choices for curative resections in patients with ulcerative colitis who require surgical removal of the colorectum^[1,2]. However, the majority of patients with curative resection subsequently develop complications including pouchitis which is the most frequent long-term complication after IPAA^[2-4]. Pouchitis was first reported by Kock *et al*^[5] in 1977 as a non-specific acute inflammation of the ileal reservoir in patients who had undergone proctocolectomy. Since then, pouchitis has become a widely recognized complication of restorative proctocolectomy.

The etiology of pouchitis is unknown, theories range from genetic susceptibility, bacterial overgrowth, ischemia, and fecal stasis to a recurrence of ulcerative colitis in the pouch, a missed diagnosis of Crohn's disease, or possibly a novel third form of inflammatory bowel disease^[6]. Some patients with suspected pouchitis may not have inflammation of the pouch, but rather, irritable pouch syndrome. Hence, endoscopic investigations with biopsy are essential to decide whether a patient has pouchitis or not. Indeed, the more commonly used scores like the pouch disease activity index incorporate both endoscopic and histologic criteria. Furthermore, unlike common inflammatory bowel disease in which numerous controlled clinical trials have been conducted and data have become available, very limited number of controlled trials, to our knowledge, have been conducted on pouchitis^[7,8].

CASE REPORT

A 27-year-old male developed ulcerative colitis in 1992. He had been treated with conventional medications including corticosteroids. However, the ulcerative colitis had

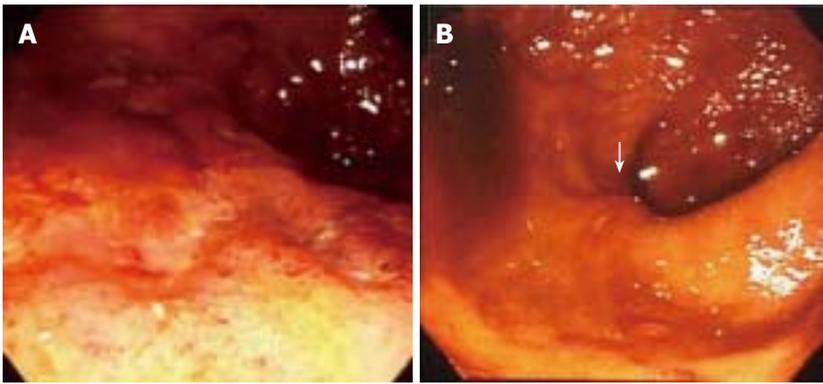


Figure 1 Endoscopic findings of refractory pouchitis. **A:** Ulcers in the ileal pouch together with inflammation, contact bleeding and mucous discharge before the treatment with rebamipide enema; **B:** Healing of ulcers in the ileal pouch with a remaining minor scar after the treatment with rebamipide enema (arrow head).

become refractory to conventional medication, and the patient therefore underwent total colectomy with mucosal proctocolectomy in 1993 and IPAA in 1994. In 1996, he presented with anal pain as well as protracted symptoms of diarrhea, abdominal pain, and incontinence which were worsening with time. The patient was diagnosed to have pouchitis and was treated with metronidazole together with betamethasone enema (3.95 mg/dose). However, despite this intensive therapy, the patient did not improve. In 2002, oral prednisolone (20 mg/dose) was given, and the patient improved for a few days. Then the patient's symptoms went into a relapsing-remitting pattern with serum C-reactive protein (CRP) levels oscillating between 50 and 10 mg/L. On endoscopy in 2003, ulceration and inflammation were seen in the ileal pouch together with contact bleeding and mucous discharge (Figure 1A). After providing written informed consent, the patient was treated with rebamipide (2-(4-chlorobenzoylamino-3-[2 (1H)-quinolinon-4-yl]-propionic acid) enemas (150 mg/dose) twice a day for 8 wk without additional drug therapy.

Two weeks after the rebamipide therapy, stool frequency started to decrease and fecal hemoglobin became negative. Endoscopic findings showed that ulcers in the ileal pouch had healed with no obvious inflammation (Figure 1B). The effect of rebamipide enema was very dramatic and was maintained throughout the therapeutic period and the 11-mo follow-up. The patient continued to be in remission. No adverse effects were observed during the treatment or the follow-up period.

DISCUSSION

Although a recent Cochrane analysis had difficulty in identifying evidence-based support, the antibiotics, metronidazole and ciprofloxacin, are often used as the first-line treatment for pouchitis^[4,6,9]. Some patients respond to this regimen and a smaller fraction respond to conventional medications with aminosalicylates, corticosteroids and immunomodulators. However, a proportion of patients with chronic pouchitis does not respond to any of these therapies and represent a major challenge to physicians. Thorough endoscopy reveals that mucosal damage (inflammation and ulcers) in refractory pouchitis and ulcerative colitis is similar. Therefore, substances that have both anti-inflammatory activity and promote epithelial cell growth (restore the integrity of the colonic mucosa and maintain its barrier function)

should be effective in refractory pouchitis. In line with this thinking, we came to know a drug called rebamipide with striking potency to promote the epithelial cell growth factor (EGF) activity and EGF-receptor expression^[10]. This prompted us to test its efficacy in a case of severe pouchitis, which did not respond to intensive conventional medication including antibiotics and corticosteroids.

The use of metronidazole in pouchitis is intended to inhibit colonization of the pouch by the bacteria which are suspected to be associated with the perpetuation of the disease in the pouch, as described for patients with an intact large bowel^[11]. However, when the disease does not respond to metronidazole and conventional therapy for inflammatory bowel disease, it may respond to a novel medication like the response to rebamipide by the present refractory case. Rebamipide has a proven anti-ulcer effect in animal models of colitis^[12]. Recently, it has been proposed that rebamipide activates in gastric epithelial cells a genetic program that promotes angiogenesis and signals cell growth and tissue regeneration^[12,13].

Thus, both the actions of rebamipide, such as the anti-ulcer effect and the activation of epithelial cell regeneration, may be attributed to the efficacy in the present case of severe pouchitis.

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Different outcomes of nosocomial infection with hepatitis C virus from the same origin

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Abstract

The outcome of infection with hepatitis C virus (HCV) varies substantially from self-limiting infection to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma among the individuals. The mechanisms that determine the clearance or the persistence of HCV have not yet been clarified. Here, we experienced two cases of hospital-related infection with HCV from the same origin but with quite different outcomes. One case resolved after an episode of acute hepatitis, while the other case developed a chronic hepatitis although they were infected with HCV of the same origin. Although infected with the virus of the same origin, the clinical and virological courses were completely different. This suggests that host factors play a major role in conditioning the outcome of acute HCV infection.

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Key words: Nosocomial infection; Hepatitis C virus; HLA

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INTRODUCTION

The outcome of infection with hepatitis C virus (HCV) varies from self-remission, chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma^[1-3]. About 20% of the people

infected with HCV spontaneously clear the virus^[4]. On the other hand, the rest of the patients cannot clear the virus and suffer from chronic infection^[4]. The mechanisms that determine the clearance or the persistence of HCV have not yet been elucidated. Viral factors such as genotype could be involved in the outcome of the infection^[5]. However, what determines the outcome of HCV infection is not totally clear. Here, we experienced two cases of hospital-related infection with HCV from the same origin but with two different outcomes. One case resolved subsequent to an acute hepatitis and the other case became a chronic hepatitis although they were infected with the same viral origin according to viral sequencing. This implies that host factors play a major role in conditioning the outcome of acute HCV infection.

CASE REPORT

A 32-year-old woman (patient 1) and a 71-year-old man (patient 2) were admitted to the same floor of Gunma University Hospital on December 2001. Clinical, biochemical, and serologic profile of patients 1 and 2 are shown in Figure 1.

Patient 1 was diagnosed with idiopathic interstitial pneumonia on February 2001 and followed up as an out patient. She was complicated by a bacterial respiratory infection with dyspnea and she was readmitted on December 21. She showed normal aminotransferase level and was negative for hepatitis B surface antigen (HBsAg) and hepatitis C antibody (HCVAb) on admission. The respiratory infection was treated with antibiotics and the patient gradually improved. Although there were no typical acute hepatitis-like symptoms except for appetite loss, the patient's serum aminotransferase level was elevated during a routine check-up on February 28, 2002. Anti-hepatitis A (HA) IgM antibody, HBsAg and hepatitis B core (HBc) IgM antibody were negative. However, HCVAb became positive at this time. HCV RNA was also positive (850 KIU/mL) and genotype was 1b. Peak level of aspartate aminotransferase (AST) was 1199 IU/L, alanine aminotransferase (ALT) was 1348 IU/L on March 25, respectively. Aminotransferase level became normal on April 15 and continued at a normal level. HCV RNA became negative on April 6 and continued to be negative. She was diagnosed with acute hepatitis C and finally recovered.

Patient 2 was first diagnosed with diabetes mellitus at the age of 44. He was treated with insulin and subsequently admitted for the control of blood sugar on December

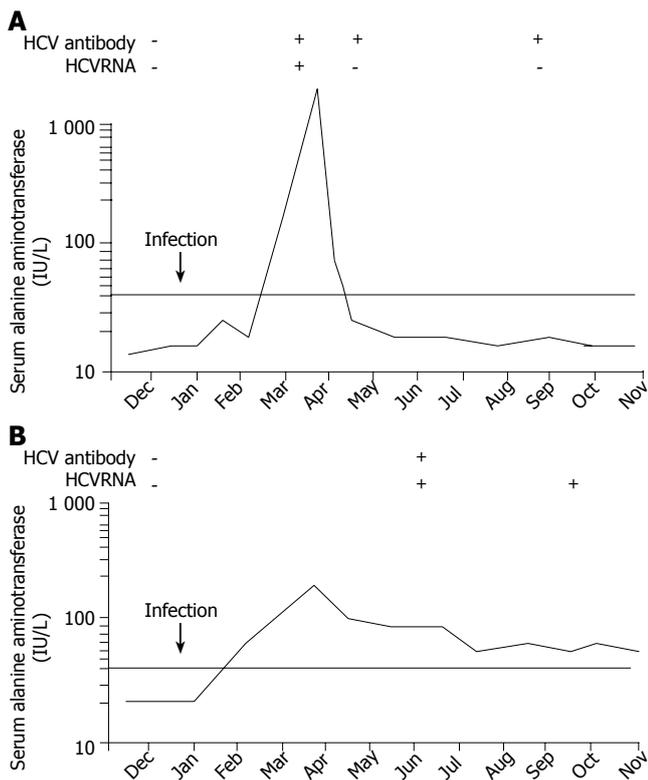


Figure 1 Clinical, biochemical, and serologic profile of patients. **A:** patient 1; **B:** patient 2. HCV antibodies were detected by third-generation tests and HCV RNA was detected in serum by reverse-transcription PCR. Alanine aminotransferase values are shown on a logarithmic scale, and the horizontal lines indicate the upper limit of the normal range.

18, 2001. At the age of 69, he was diagnosed with myelodysplastic syndrome. Upon admission aminotransferase levels were normal, and both HBsAg and HCVAb were negative. He was discharged on January 8, 2002 and followed up with his primary physician. He subsequently developed elevated aminotransferase levels and became positive for HCVAb in June 2002. There were no typical acute hepatitis-like symptoms during the follow-up period with his primary physician. Retrospectively, the aminotransferase level of the patient was elevated during a routine check-up in January 31, 2002. HCV RNA was also positive (250 KIU/mL) and genotype was 1b. Peak level of AST was 102 IU /L, ALT was 168 IU /L on March 28. Aminotransferase levels continued to be abnormal for 3 years after the onset. He was diagnosed with chronic hepatitis C.

We believe that HCV infection of both patients occurred during December 2001 to January 2002. There was no suspicious event in the history of the patient suggesting infection such as intravenous drug abuse, blood transfusion, tattoo nor transmission by sexual contact in both cases. Nosocomial infection was suspected and surveyed. HCV genotyping and the nucleotide sequence analysis of coding region for the envelope glycoprotein E1 were performed for the two patients. For the nucleotide sequence analysis, after reverse transcription, the first round of polymerase chain reaction used primers EF1 (sense: 5'-CGCCGACCTCATGGGTA-3', nt 721 to 739) and ER1 (antisense: 5'-CGACCAGTTCATCATCATATCCCA-3', nt 1 289 to 1 313), and the second-round primers EF2 (sense: 5'

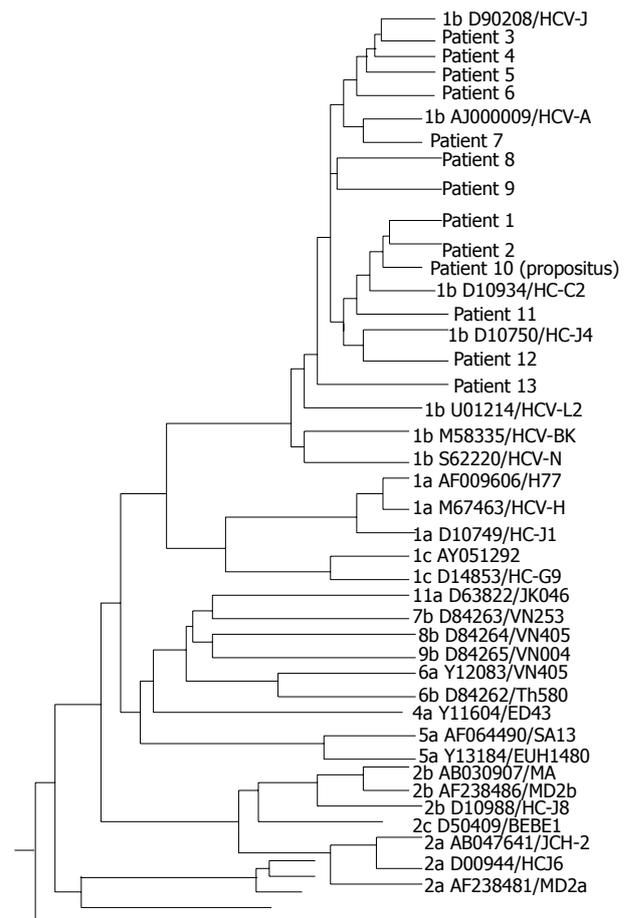


Figure 2 Phylogenetic tree analysis comparing coding sequences in the HCV regions for the envelope glycoproteins E1. For phylogenetic tree analysis, 13 sequences obtained from the 13 patients involved in this study were compared with 33 sequences taken as unrelated controls (genotype and GenBank accession numbers are indicated).

-TTGCCCGGTTGCTCTTTTTCTATC-3', nt 837 to 860) and ER2 (antisense: 5'-GCGGTGACCTGATACATGGC-3', nt 1 264 to 1 283). We performed direct-sequence analysis of nested polymerase chain reaction products of 447 bp (nt 837 to 1 283) encompassing the HCV envelope glycoprotein E1. The polymerase chain reaction products were gel purified and sequenced by automated sequencer. The HCV genotype of both patients was 1b. The sequences of HCV E1 region from both the patients were 99.7% in agreement. We considered that the origins of both infections were the same and these two hepatitis infections were hospital related. We checked up on all the patients who were admitted to the same floor from December 18, 2001 to January 8, 2002, the suspicious infection period in question. There were 16 patients positive for HCV antibody. These 16 patients were examined for the genotype of HCV and 11 of them were 1b. The nucleotide sequences of E1 region in these 11 patients (numbered patient 3-13) were compared by the same method. Phylogenetic tree analysis comparing coding sequences in the HCV E1 region are shown in Figure 2. For phylogenetic tree analysis, 13 sequences obtained from the 13 patients involved in this study were compared with 33 sequences taken as unrelated controls. One patient had close nucleotide sequenc-

ing with patients 1 and 2. A 70-year-old man (patient 10, named as propositus) with liver cirrhosis was considered as the propositus of this hospital-related infection of HCV. He was admitted for treatment of hepatic encephalopathy from December 26-29.

Thus, patient 1 had a complete resolution of acute hepatitis C, while patient 2 developed a chronic hepatitis C infection although both were infected by the same HCV strain (propositus, patient 10). Although it was not fully elucidated how the nosocomial infection occurred, intravenous catheter flushing with heparin retrieved from a multidose heparin solution in saline was thought to be one of the causes of the infection.

DISCUSSION

Although the same HCV strain infected two patients, each patient showed a completely different outcome. One case was cured from acute hepatitis and the other case developed chronic hepatitis although they were infected with HCV of the same origin. Primary HCV infection is poorly characterized because most patients are asymptomatic and, therefore, it is rarely diagnosed^[3,6,7]. Larghi *et al.*^[8] reported the outcome of an outbreak of acute hepatitis C supposedly infected from a common source. Among the 14 patients followed up, 8 patients resolved spontaneously and 6 patients developed chronic infection^[8]. The incubation period and the outcome of the acute phase of the disease were highly variable. The average incubation period was 10 wk, but the range was from 6 to 28 wk. In our case, the supposed incubation periods of patients 1 and 2 were 9 and 5 wk, respectively.

The clinical and virologic course also varied, both in patients in whom the infection resolved spontaneously and in those developing chronic infection^[8]. This suggests that host factors play a major role in conditioning the outcome of acute HCV infection^[9,10]. Thus, the host immune system is important for the clearance of virus. In view of the host factors, specific MHC class II alleles were reported to influence susceptibility or resistance to persistent HCV infection^[10,11]. The associations of self-limiting HCV infection with HLA-DRB1*1101 and HLA-DQB1*0301 have been independently reported by some groups^[10,11]. Persistent HCV infection was associated with HLA-DRB1*0701, and HLA-DRB4*0101. In our case, HLA was not fully evaluated in all the patients. However, propositus had DRB1*0901, DRB1*1502, DQB1*0303, and DQB1*0601. Patient 2 had DRB1*0901, DRB1*1403, DQB1*0303, and DQB1*0301. HLA-DQB1*0301 was not a self-limiting factor in our case.

We were able to determine the propositus of the HCV infection; however, the route of infection was not fully understood. There was no procedure identified in these patients to directly cause the infection, such as endoscopy, surgery or hemodialysis. Finally, flushing of intravenous catheters with heparin retrieved from a

multidose heparin solution in saline was thought to have caused the infection. Since January 2002, our department has been using heparin solution packed for individual use for flushing of intravenous catheters. The period of this nosocomial infection was before the use of individual packed heparin solution was instituted. All had long-lasting intravenous catheters. Multidose vials used for flushing or treatment had probably been contaminated during periods of overlapping treatment. Contamination of multidosing vials was the most likely mode of HCV transmission^[12]. Therefore, use of such vials should be avoided.

We experienced two cases of hospital-related infection of HCV from the same origin followed by different outcomes. One patient was cured of acute hepatitis and the other case developed chronic hepatitis although both were infected by the same propositus. Although they were infected by the same source, the clinical and virologic course was completely different. This suggests that the host factors play a major role in determining the outcome of acute HCV infection.

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CASE REPORT

Left-sided omental torsion with inguinal hernia

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Abstract

We report a case of surgically proved left-sided torsion of the greater omentum that caused secondary by untreated inguinal hernia. Case A 36-year-old man presented to our hospital with abdominal pain. He had been diagnosed with a left inguinal hernia, but he had not received any treatments. Contrast-enhanced computed tomography (CT) of the abdomen showed a large fat density mass below the Sigmoid colon and left inguinal hernia with incarcerated fat. Exploratory laparotomy revealed torsion of the greater omentum with small bloody ascites. The greater omentum was twisted into one and a half circles and entered into a left inguinal hernia. An omentectomy with a repair of left inguinal hernia was performed. A resected omentum was submitted for pathological examination, which showed hemorrhagic infarction. Omental torsion is a rare cause of acute abdominal pain but should be included in the differential diagnoses of acute abdomen, especially in patients with untreated inguinal hernia.

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Key words: Omental torsion; Acute abdomen; Inguinal hernia

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INTRODUCTION

Torsion of the omentum is a rare cause of acute abdomen, and twisting of the omentum may lead to subsequent

vascular impairment and eventually progress to infarction of the segment of the omentum distal to the twisting point. In most cases, torsion occurs on the right side of great omentum and left-side torsion is infrequent^[1,2].

We have reported a case of surgically proved left-sided torsion of the greater omentum that caused secondary torsion by untreated inguinal hernia.

CASE REPORT

A 36-year-old man presented to our hospital with abdominal pain of sudden onset. He had been diagnosed with a left inguinal hernia, but he had not received any treatment. Physical examination revealed tenderness in the left lower quadrant of the abdomen, but left inguinal swelling was not present. Blood examination showed leukocytosis (white blood cell count = 14 700/mm³), and the C-reactive protein (CRP) level was elevated (3.84 mg/dL). Plain abdominal X-rays showed a right upper deviation of the sigmoid colon. Contrast-enhanced computed tomography (CT) of the abdomen showed a large fat density mass below the sigmoid colon and left inguinal hernia with incarcerated fat (Figure 1). Conservative therapy including intravenous administration of antibiotics was performed. However, his symptoms were not resolved and a small collection of free fluid was noted within the peritoneal cavity by CT. Exploratory laparotomy was performed on March 29, 2004, which revealed torsion of the greater omentum with small amount of bloody ascites. The greater omentum was twisted into one and a half circles and entered into a left inguinal hernia (Figure 2). An omentectomy with a repair of left inguinal hernia was performed. A resected omentum was submitted for pathological examination, which showed hemorrhagic infarction. Post-operative course was uneventful, and the patient has remained healthy with no clinical evidence of recurrent disease since then.

DISCUSSION

Torsion of the omentum is a rare cause of acute abdomen and the majority of cases have right-side torsion. Left-side torsion is extremely rare and there are only sporadic reports in the literature^[1,2]. The greater susceptibility to torsion of the right side is due to its greater length and size in relation to the left side and its greater mobility^[3].

Omental torsion can be classified as primary and secondary torsion, the latter being more common. Primary



Figure 1 Contrast-enhanced computed tomography showing a large fat density mass below the sigmoid colon and left inguinal hernia with incarcerated fat (arrow).

torsion is believed to be related to local omental anomalies, such as bulky bifid or accessory omentum or abnormally redundant omental veins, but the exact pathogenesis is yet to be uncovered^[4]. Secondary torsion is associated with adhesions to cysts, tumors, inflammatory foci, scars, or hernias. The precipitating factors are shared in both omental torsions, which include sudden increase in intra-abdominal pressure following a heavy meal or exertion, change in body position, coughing or sneezing, and occupational use of vibrating tools^[2,3]. These events may result in sudden displacement of the omentum, thus exposing the omentum to greater risk of torsion. Torsion leads to omental infarction and usually presents as acute abdominal pain.

The majority of the cases of omental torsion are torsion involving the right-side of the omentum with the right lower quadrant or right paraumbilical pain. Thus, the differential clinical diagnoses of omental torsion include acute appendicitis, acute cholecystitis, cecal diverticulitis, or appendagitis. Left-sided omental torsion as in our patient is unusual and clinically mimics sigmoid diverticulitis, or appendagitis.

Diagnosis of omental torsion is rarely made preoperatively but should become more frequent with the increasing use of CT in the diagnosis of acute abdominal conditions^[5]. The CT findings of fatty mass with a whirling pattern in the greater omentum suggest omental torsion. Although this typical CT finding has been documented in literature from various countries^[6,7], it should be made clear that not all omental torsions show this relatively characteristic appearance as in our case. The whirling pattern may not be apparent in cases where the axis of rotation is not perpendicular to the transverse scanning plane.

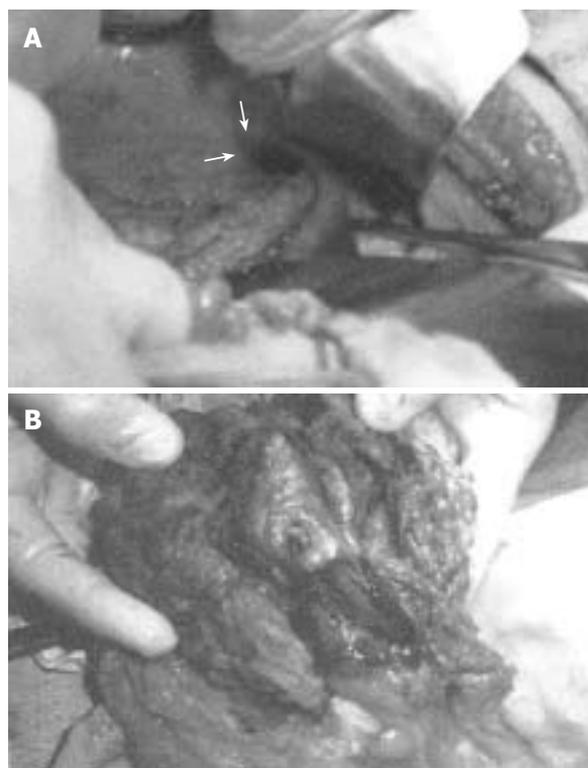


Figure 2 Twisting of greater omentum into one and a half circles and entering into a left inguinal hernia (arrow).

Concerning the treatment of omental torsion, resection of the involved segment of the omentum has traditionally been the treatment of choice^[2]. However, a few reported cases of omental torsion that were successfully treated conservatively have been reported^[2,5], especially in patients without associated complications. This may suggest that surgical treatment should be limited to the patients with complications. However, in cases where conservative treatments are not effective, as in our case, surgical treatments should be considered.

In conclusion, omental torsion is a rare cause of acute abdominal pain but should be included in the differential diagnoses of acute abdomen, especially in patients with untreated inguinal hernia. It is necessary to consider radiographic findings to determine how to best manage these patients and treat them.

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Coexistence of hepatocellular carcinoma and gastrointestinal stromal tumor: A case report

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Abstract

Malignant gastrointestinal stromal tumors (GIST) are rare mesenchymal tumors originating from the wall of the gastrointestinal tract. Their coexistence with other tumors originating from other germ layers is unique. We have reported a case of a 63-year-old GIST patient presenting as an epigastric mass associated with hepatic tumor. Histologically, the mesenteric tumor was composed of spindle cells showing both neural and smooth muscle differentiation. Immunohistochemical examination showed positive staining for CD117, vimentin, S-100, and SMA, while CD34 antigen was negative. The hepatic tumor was diagnosed as hepatocellular carcinoma (HCC). To the best of our knowledge, this is the first case of GIST and HCC coexistence. The rarity of the case, however, should not lead to ignoring such a possibility in differential diagnosis.

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Key words: Malignant GIST; Hepatocellular carcinoma; Coexistence of GIST and HCC

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INTRODUCTION

Malignant gastrointestinal stromal tumors (GIST) are rare mesenchymal tumors originating from the wall of the gastrointestinal tract^[1,2]. Majority of GIST are positive for

CD117 and vimentin in immunohistochemical staining^[3-5]. Coexistence of malignant GIST and other malignancies have been reported in some unique cases^[6-10]. Such cases always require differential diagnosis for metastatic GIST^[3,4]. We have described a case of a 63-year-old male patient who was admitted to our clinic with liver tumor mass and mesenteric tumor. Preoperative biopsy performed under computed tomography (CT) scanning showed a mesenteric tumor as GIST, which was confirmed by postoperative histopathology. The liver tumor was verified as hepatocellular carcinoma (HCC). This case presents a coexistence of two independent tumors. To our knowledge, the coincidence of GIST and HCC has not been reported in the literature.

CASE REPORT

A 63-year-old male patient with a palpable epigastric mass was admitted to the Department of Surgical Oncology, Medical University of Gdansk. His medical history was significant for insulin dependent diabetes mellitus and ischemic heart disease. No other clinical symptoms were present. The morphology, serum alpha-fetoprotein (AFP), liver enzymes and other blood tests were normal.

CT of the patient's abdomen showed an 80 mm×50 mm pathological mass in the right side of abdomen and a 65 mm×53 mm solid mass within the liver (Figures 1A and 1C). Abdominal sonography showed a 80-mm mass in diameter in the mesentery at the right side of the abdomen with mediocre flow in Doppler option and a 60-mm solid mass in diameter in the left lobe of the liver with low flow in Doppler option (Figures 1B and 1D). Laparotomy revealed a mesenteric tumor on the intestinal wall and a hepatic tumor of the same size as in ultrasonographic description (Figures 2A and 2B). No other malignant pathologies in the abdomen were found. Left hemihepatectomy and resection of mesenteric tumor with side-to-side jejunojejunostomy were performed.

Histologically, the liver tumor was described as HCC. The mesenteric tumor 70 mm×80 mm×60 mm in size was composed of spindle cells showing both neural and smooth muscle differentiation. Resection margins were histologically negative. Immunohistochemically, the tumor cells were positive for CD117, vimentin, SMA, S-100, and negative for CD34. Mitotic index was 10/50 HPF (10 pathological mitoses per 50 high-power field). The final pathological diagnosis of this tumor was gastrointestinal stromal tumor (GIST). The patient was discharged 9 d

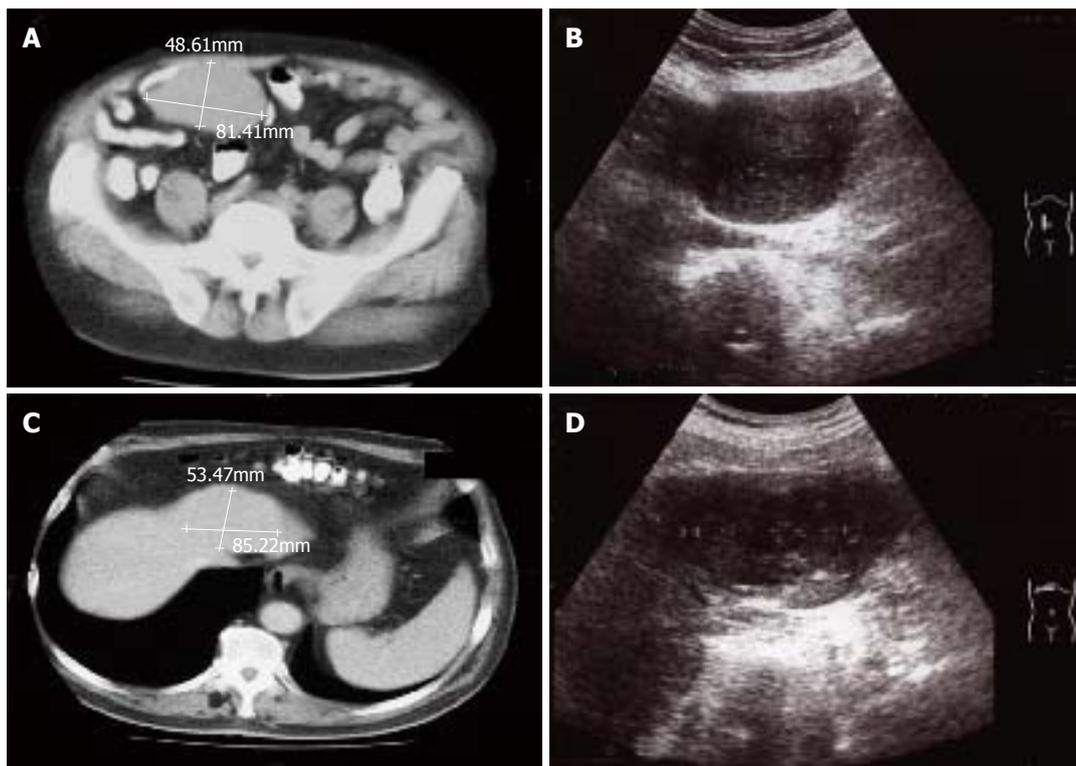


Figure 1 Computed tomography and sonography of mesenteric mass (A and B) and liver tumor (C and D).

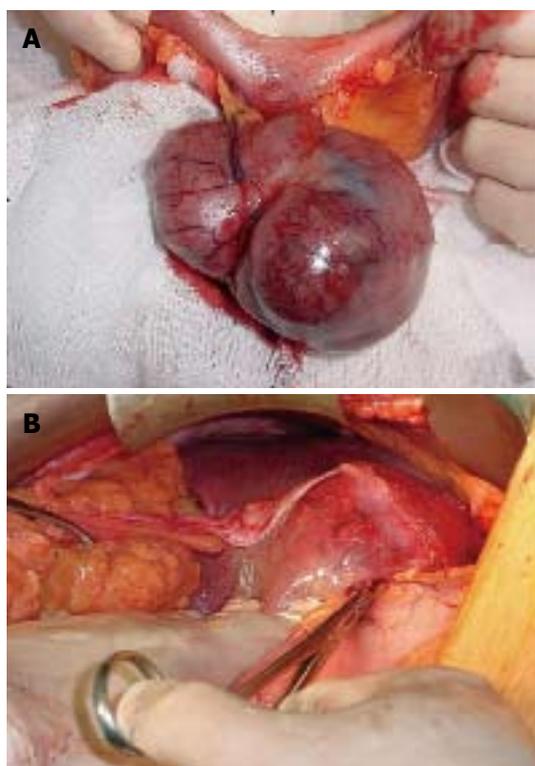


Figure 2 Mesenteric (A) and hepatic (B) tumors (intraoperative view).

after tumor resection without surgical complications. No adjuvant therapy was prescribed, because the liver tumor was not metastasized from GIST. The patient after 18 mo of follow-up showed no signs of recurrent disease.

DISCUSSION

GIST are rare tumors that may arise from anywhere in the tubular gastrointestinal tract and give rise to metastases predominantly in the liver (more than 60% of metastases) or in the peritoneum^[1,2,4,11,12]. In the present case, the solid mass in the liver was metastasized from the GIST. The histopathology showed that the tumor was a HCC. Coexistence of GIST and other primary tumors: renal carcinoma, bilateral pheochromocytoma, gastric cancer, and leiomyoma of the anorectum has been reported^[6-10].

GIST may coexist also with inflammatory pseudotumors of the liver. These lesions are benign and characterized by proliferating fibrovascular tissue with inflammatory cells. They are associated with fever, pain and a mass effect. These clinical symptoms were not observed in our patient. The inflammatory pseudotumors are commonly mistaken for malignant tumors^[13].

The described case is unique and should draw physicians' attention to the possibility of independent tumor coexistence when GIST occurs. In patient with two tumors in the abdomen, GIST and HCC coexistence must be excluded. This is especially important in making a decision of adjuvant therapy. The current GIST treatment guidelines established by European Society for Medical Oncology (ESMO) for adjuvant therapy with imatinib can be applied in non-operable and/or metastatic tumors. Patients with resectable tumors without metastases should not receive adjuvant therapy. The radiotherapy value as an adjuvant treatment method for GIST is not documented in the literature^[5].

The presence of hepatic tumor in patients with GIST should not judge the metastatic nature of liver tumors,

though in the literature it is the most common cause of the coexisting liver tumor. Although two independent tumors in patients with GIST are unique, we must not exclude such a possibility and only careful histopathological examination gives us a correct diagnosis.

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Gastroenterology Association
May 20-25, 2006
Loas Angeles Convernition Center, California
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EVENTS AND MEETINGS IN THE UPCOMING 6 MONTHS

10 th World Congress of the International Society
for Diseases of the Esophagus (ISDE 2006)
February 22-25, 2006
Adelaide
isde@sapmea.asn.au
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EASL 2006 - The 41st Annual Meeting
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Vienna, Austria

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Los Angeles, CA
www.asge.org/education

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| 22 | 10 μM | 10 μmol/L | |
| 23 | 1N HCl | 1 mol/L HCl | |
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| 32 | 1 pg L ⁻¹ | 1 pg/L | |
| 33 | 10 kilograms | 10 kg | |
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| 35 | 1000 g | 1 000 r/min | Low speed. g cannot be used. |
| 36 | Gene bank | GenBank | International classified genetic materials collection bank |
| 37 | Ten L | Ten liters | At the beginning of a sentence |
| 38 | Ten mL | Ten milliliters | At the beginning of a sentence |
| 39 | umol | μmol | |
| 40 | 30 sec | 30 s | |
| 41 | 1 g/dl | 10 g/L | 10-fold conversion |
| 42 | OD ₂₆₀ | A ₂₆₀ | "OD" has been abandoned. |
| 43 | One g/L | One microgram per liter | At the beginning of a sentence |
| 44 | A260 nm ^b P<0.05 | A ₂₆₀ nm ^a P<0.05 | A should be in italic. In Table, no note is needed if there is no significance instatistics: ^a P<0.05, ^b P<0.01 (no note if P>0.05). If ther is a second set of P value in the same table, ^c P<0.05 and ^d P<0.01 are used for a third set: ^a P<0.05, ⁱ P<0.01. |
| 45 | [*] F=9.87, [§] F=25.9, [#] F=67.4 | ¹ F=9.87, ² F=25.9, ³ F=67.4 | Notices in or under a table |
| 46 | KM | km | kilometer |
| 47 | CM | cm | centimeter |
| 48 | MM | mm | millimeter |
| 49 | Kg, KG | kg | kilogram |
| 50 | Gm, gr | g | gram |
| 51 | nt | N | newton |
| 52 | l | L | liter |
| 53 | db | dB | decibel |
| 54 | rpm | r/min | rotation per minute |
| 55 | bq | Bq | becquerel, a unit symbol |
| 56 | amp | A | ampere |
| 57 | coul | C | coulomb |
| 58 | HZ | Hz | |
| 59 | w | W | watt |
| 60 | KPa | kPa | kilo-pascal |
| 61 | p | Pa | pascal |
| 62 | ev | EV | volt (electronic unit) |
| 63 | Jonle | J | joule |
| 64 | J/mmol | kJ/mol | kilojoule per mole |
| 65 | 10×10×10cm ³ | 10 cm×10 cm×10 cm | |
| 66 | N·km | KN·m | moment |
| 67 | x±s | mean±SD | In figures, tables or text narration |
| 68 | Mean±SEM | mean±SE | In figures, tables or text narration |
| 69 | im | im | intramuscular injection |
| 70 | iv | iv | intravenous injection |
| 71 | Wang et al | Wang <i>et al.</i> | |
| 72 | EcoRI | EcoRI | <i>Eco</i> in italic and RI in positive. Restriction endonuclease has its prescript form of writing. |
| 73 | Ecoli | <i>E.coli</i> | Bacteria and other biologic terms have their specific expression. |
| 74 | Hp | <i>H pylori</i> | |
| 75 | Iga | Iga | writing form of genes |
| 76 | igA | IgA | writing form of proteins |
| 77 | ~70 kDa | ~70 ku | |

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Novel mechanisms in functional dyspepsia

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Abstract

Functional dyspepsia (FD) is a highly prevalent but heterogeneous disorder in which multiple pathogenetic mechanisms are involved. Although there are many studies that have investigated various pathophysiologic mechanisms, the underlying causal pathways associated with FD remain obscure. The currently proposed pathophysiologic mechanisms associated with FD include genetic susceptibility, delayed as well as accelerated gastric emptying, visceral hypersensitivity to acid or mechanical distention, impaired gastric accommodation, abnormal fundic phasic contractions, abnormal antroduodenal motility, acute and chronic infections, and psychosocial comorbidity. A greater understanding of the abnormalities underlying FD may lead to improved management. The aim of this editorial is to provide a critical overview of current pathophysiologic concepts in functional dyspepsia.

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Key words: Functional dyspepsia; Gastric function; Pathophysiology

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INTRODUCTION

Dyspepsia, or pain or discomfort centered in the upper abdomen, is a common condition accounting for 2-5% of all primary care consults; the annual prevalence is 25%^[1-3]. Dyspepsia is a major cause of morbidity and economic loss in the community^[4]. A heterogeneous group of pathophysiologic mechanisms have been implicated in the etiology of functional dyspepsia (FD).

Delayed gastric emptying^[5], antral hypomotility and altered intestinal motility^[6], diminished gastric accommodation^[7], *Helicobacter pylori* infection^[8], abnormal duodenal sensitivity to acid^[9], enhanced visceral sensitivity^[10], carbohydrate maldigestion^[11], food intolerance^[12], and psychological factors^[13] have all been identified in subgroups of patients with functional dyspepsia, with much overlap.

Despite the high prevalence of FD and its significant impact on social and health care costs, the underlying causal pathways are still obscure. Therefore, our objective here is to briefly provide an overview of the known pathophysiology of FD, and highlight recent advances in our knowledge.

GENETICS

Recent evidence supports the relevance of a genetic milieu in FD. A case-control study by Holtmann *et al*^[14] suggested that there is a significant link between GN β 3(C825T) CC genotype and functional dyspepsia (OR = 2.2, 95% CI = 1.4-3.3). Although their study^[14] had some limitations including the high prevalence of the CC genotype among control subjects and the relatively modest excess of the genotype in FD subjects, their findings do represent the first identification of a putative genetic predisposition. The association has been independently confirmed^[15]. Further studies of the relationship between genetic polymorphisms and other pathophysiologic factors in FD are now needed.

ABNORMALITIES IN UPPER GUT MOTOR FUNCTION

A prevalence of disturbances in gut motor function of about 20-40% in FD patients is now accepted^[6,7,16]. The disturbances of gastric motor function most often reported in FD include delayed gastric emptying^[16], abnormalities in antral contractility^[6] and an impaired accommodation response (a vagally-mediated reflex relaxation of the fundus in response to a meal)^[7].

Gastric emptying

Delayed gastric emptying has been described in 25-50% of patients with FD^[16-19]. The majority of small sample sized investigations failed to identify association between delayed gastric emptying and any dyspeptic symptoms^[20-22]. Stanghellini *et al*^[16], in a large study with 343 patients with FD, showed that female sex, relevant and severe postprandial fullness, and severe vomiting were independently associated with delayed gastric emptying of solids. Other European studies have generally confirmed

these findings^[17,18], but studies from the US have not^[23,24]. Recently, accelerated gastric emptying in FD has also been associated with postprandial fullness, bloating, nausea, and pain, presumably through an early dumping syndrome^[25]. These findings imply that administration of a prokinetic agent to accelerate gastric emptying may actually lead to worsening, rather than to improvement of symptoms, in at least a proportion of patients with FD.

Impaired gastric accommodation

Gastric accommodation is a vagally mediated reflex that occurs post-prandially, results in reduction of tone, and provides a reservoir for the meal; it enables the stomach to handle increases in intragastric volume without proportional increases of intragastric pressures^[7,26,27].

Studies by the barostat, scintigraphy, ultrasonography, single photon emission computed tomography (SPECT), and non-imaging assessments (satiating drinking test) have all suggested abnormal accommodation in up to 40% of patients with functional dyspepsia^[7,26-30]. The mechanism of impaired gastric accommodation is, however, not clearly understood. Tack *et al*^[26] proposed that impaired proximal stomach accommodation of an ingested meal was associated with early satiety and weight loss in FD, but other studies have not confirmed these symptom associations^[24,28].

Relaxation of the proximal stomach can be activated by duodenal distension or nutrient infusion, or via a vagovagal reflex pathway, and it requires activation of intrinsic nitrergic neurons in the stomach. Recent reports^[31,32] have suggested that a defect at the level of the gastric intrinsic nitrergic neurons may be relevant. Abnormal vagal reflexes may also play a role, because postvagotomy patients have a similar impairment of gastric accommodation, and abnormal vagal function is observed in patients with FD^[33,34].

Abnormal phasic contractility of the proximal stomach

Phasic fundic contractions induce transient increases in gastric wall tension and can be perceived in FD^[8,35]. In the recent study using a gastric barostat, unsuppressed postprandial phasic activity in the proximal stomach was present in a small subgroup of patients with FD^[36]. This was associated with symptoms of severe postprandial bloating. However, the mechanisms underlying unsuppressed phasic contractility are unclear and further studies will be required.

Abnormalities of gastric myoelectrical rhythm

Electrogastrography obtains a cutaneous recording of gastric myoelectrical activity from abdominal surface electrodes. Some studies^[37-39] have reported gastric myoelectrical abnormalities are found in up to two-thirds of patients with FD. A close relationship between the presence of delayed gastric emptying and abnormalities of gastric electrical rhythm has been reported^[37-39]. However, their relevance to symptom generation is still uncertain.

Antro-duodenal dysmotility

Manometric studies have demonstrated antral hypomotility in a large proportion of those with FD^[27,40,41].

Antral hypomotility could be associated with gastric stasis manifested by a slower emptying rate of meals^[40], but its correlation with specific symptoms is poor.

DISTURBANCES IN VISCERAL SENSITIVITY

The gut wall contains three kinds of neural receptors: chemoreceptors in the mucosa, which respond to chemical stimuli; mechanoreceptors in the smooth muscle layer, which respond to stretch or compression; and nociceptors, the most numerous receptors in all layers, which are commonly silent, but can be recruited by any stimulus strong enough to induce pain^[42].

Visceral hypersensitivity to mechanical distension

Increased perception to visceral physiological or minor noxious stimuli, i.e., a lower threshold for sensation, has been considered one of major pathophysiologic mechanisms in the functional gastrointestinal disorders^[28,43-44]. The level of abnormality where visceral hypersensitivity is located is not clear, but several observations suggest that alterations exist at the level of the central nervous system or in addition to hyperexcitability of visceral afferents^[43-46].

Several studies have found dyspeptic patients have lower thresholds for first perception and discomfort or pain during isobaric gastric distension or jejunal distension^[29,47,48]. Mertz *et al*^[44] reported that hypersensitivity to gastric balloon distension was specific for FD, and Holtmann *et al*^[49, 50] have shown a failure of sensory thresholds to increase in patients with FD. While Tack *et al*^[43] have shown hypersensitivity to gastric distension was associated with a higher prevalence of post-prandial pain, belching and weight loss. The symptom correlations remain to be confirmed.

Abnormal central nervous system processing of afferent information during gastric stimuli has been suggested to be one of the mechanisms of visceral hypersensitivity^[46]. Ladabaum *et al*^[51] performed PET scanning during distal gastric distension in healthy volunteers, and found increases in activation of somatic and visceral pain processing areas that were proportional to the distension-evoking stimuli. Vandenberg *et al*^[52] also performed PET scanning after proximal gastric distension in healthy volunteers, and found a neuronal network processing distension stimuli from the proximal stomach. Further study is needed to assess whether abnormalities in central processing of visceral stimuli occur in FD.

Visceral hypersensitivity to chemical stimuli

Several studies^[53-55] have been conducted to evaluate chemical sensitivity in FD, but its mechanism is not clear. In both healthy subjects and in patients with FD, perception of gastric distension was enhanced by nutrient lipids but not by glucose^[55]. Feinle *et al*^[56,57] studied the effects of lipid on gastrointestinal sensation in healthy and dyspeptic subjects and suggested that cholecystokinin A (CCK-A) and serotonergic (5-HT₃) receptors mediate, at least in part, gastrointestinal sensation. They reported that intravenous administration of a CCK-A receptor antagonist reduced the effects of duodenal lipid on gastric

relaxation and gastrointestinal sensations during gastric distension. Although they studied only small numbers of patients, these findings support changes in chemical sensitivity in FD; this needs to be validated in larger studies.

In a subset of FD patients, but not in healthy controls, intraduodenal infusion of hydrochloric acid (HCl) was found to induce nausea^[9], suggesting duodenal hypersensitivity to acid. Some patients had impaired clearance of acid from the duodenum and impaired duodenal motor response to acid. Recently, Tack *et al*^[58] observed that duodenal acidification increased proximal gastric mechanosensitivity, induced proximal gastric relaxation, and inhibited proximal gastric accommodation to a meal. However, the underlying mechanisms remain to be elucidated in future studies.

INFECTIOUS AGENTS

Acute and chronic infections may be involved in the pathogenesis of the functional bowel disorders^[31,59-61].

Helicobacter pylori infection

Many studies and several meta-analysis have tried to establish a relationship between *Helicobacter pylori* (*H pylori*) infection and FD^[59-63]. The most recently published meta-analysis^[64] suggests that *H pylori* eradication at 12 months has a small but statistically significant benefit in the treatment of FD (relative risk of remaining dyspeptic with *H pylori* eradication therapy = 0.91; 95% CI = 0.87-0.99). While statistically significant, the clinical significance of this finding is less clear because the effect is small, that is, 15 *H pylori*-positive dyspeptic patients will need to be treated to achieve just one cure.

The association between *H pylori* infection and pathophysiologic mechanisms has been investigated; however, no consistent differences in the prevalence and severity of individual symptoms, gastric emptying rate, or gastric relaxation after a meal were found between *H pylori*-positive and *H pylori*-negative subjects^[60,61].

Post-infectious dyspepsia

It has now been well established that irritable bowel syndrome may follow an acute intestinal infection. Post-infectious dyspepsia has been recognized as a possible clinical entity by Tack *et al*^[31]. In a large retrospective, tertiary referral center study, they showed that a subset of dyspeptic patients has a history suggestive of post-infectious dyspepsia. Compared with patients with unspecified-onset dyspepsia, patients with presumed post-infectious dyspepsia have more prevalent symptoms of early satiety, weight loss, nausea, and vomiting and had a significantly higher prevalence of impaired accommodation of the proximal stomach, whereas both groups did not differ in the prevalence of delayed gastric emptying, or hypersensitivity to gastric distention^[31]. They suggested that impaired accommodation in patients with presumed post-infectious dyspepsia is attributable to a dysfunction at the level of gastric nitrergic neurons. In this study, the lack of prospective design limited the validity of the results.

In a prospective cohort study, Mearin *et al*^[65] found

that development of dyspepsia was increased 5-fold at 1 year after acute *Salmonella* gastroenteritis as compared with control subjects without baseline infection. There were some limitations because of the low response rate in the 1-year follow-up survey and lack of information on psychological status and stressful events^[65]. Further studies are needed to identify the underlying pathophysiology and risk factors, and define the long-term prognosis.

PSYCHOSOCIAL ABNORMALITIES

In FD patients, a number of psychological and personality factors have been observed, including somatization, depression, and anxiety as well as health seeking behavior and alterations in illness behavior and coping styles^[66-68]. These psychosocial factors are influenced by and influence GI symptoms; this bidirectional flow is mediated through the brain-gut axis^[43]. The value of psychological therapies in FD, to date, is promising, but further studies of psychological treatment of FD are needed^[69].

CONCLUSIONS

FD is a highly heterogeneous disorder. Traditional pathophysiological paradigms are still inadequate to explain the variation in the symptoms observed. Current symptom subclassifications have also largely failed to identify responders to specific therapies. A new classification based on mechanisms and specific symptoms needs to be considered to further advance the field. The contributors to this heterogeneity need to be better defined, and be environmental, pathological, psychological, or genetic. This could lead to better targeting of treatment in FD patients with unexplained epigastric pain or discomfort.

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REVIEW

Liver cirrhosis and arterial hypertension

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Abstract

Characteristic findings in patients with cirrhosis are vasodilatation with low overall systemic vascular resistance, high arterial compliance, increased cardiac output, secondary activation of counter-regulatory systems (renin-angiotensin-aldosterone system, sympathetic nervous system, release of vasopressin), and resistance to vasopressors. The vasodilatory state is mediated through adrenomedullin, calcitonin gene-related peptide, nitric oxide, and other vasodilators, and is most pronounced in the splanchnic area. This constitutes an effective (although relative) counterbalance to increased arterial blood pressure. This review considers the alterations in systemic hemodynamics in patients with cirrhosis in relation to essential hypertension and arterial hypertension of the renal origin. Subjects with arterial hypertension (essential, secondary) may become normotensive during the development of cirrhosis, and arterial hypertension is rarely manifested in patients with cirrhosis, even in cases with renovascular disease and high circulating renin activity. There is much dispute as to the understanding of homeostatic regulation in cirrhotic patients with manifest arterial hypertension. This most likely includes the combination of vasodilatation and vasoconstriction in parallel.

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Keywords: Arterial compliance; Central vascular filling; Chyperdynamic circulation; Kidney function, Nitric oxide; Blood pressure regulation; Renin-angiotensin-aldosterone system; Sympathetic nervous system; Vasodilatation; Vasoconstriction

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INTRODUCTION

Arterial hypertension is seldom found in patients with liver disease, but patients with alcoholic fatty liver quite often present with raised arterial blood pressure. Renal involvement is seen in hepatitis B and in certain cases this may be accompanied by arterial hypertension. Otherwise, the clinical course of most liver diseases is characterized by low arterial blood pressure. This paper focuses on the circulatory dysfunction in cirrhosis, which is the most common characteristic of the chronic liver diseases.

Abnormalities in the splanchnic circulation are well described in patients with cirrhosis^[5-7]. These include increased inflow to the splanchnic system and portal venous hypertension. Moreover, such patients are often hyperkinetic with increased heart rate and cardiac output. The hyperkinetic systemic circulation is related to the severity of cirrhosis, sodium-water retention, and presence of ascites^[8]. The overall systemic vascular resistance is decreased, the blood volume is abnormally distributed with relative and absolute central hypovolemia, and cardiovascular, renal, and other organ dysfunction is common, including abnormal neurohormonal regulation^[8-10]. Essential hypertension is frequently reported in both developed and developing countries, and the prevalence of essential hypertension in patients with cirrhosis would be expected to be around 15%^[11,12]. However, most reports put the coexistence of cirrhosis and arterial hypertension much lower. As in patients with essential hypertension, most circulatory changes in cirrhosis are dysfunctional, rather than structural.

This review considers the cardiovascular system, neurohormonal regulatory systems, kidney function, and sodium-water retention with respect to arterial blood pressure and its regulation in patients with cirrhosis with the purpose of analyzing the background of the profound circulatory changes, their development, and complications. These elements are considered with respect to essential hypertension and arterial hypertension of renal origin.

HEPATOSPLANCHNIC CIRCULATION

The mechanisms behind the circulatory changes in the hepatosplanchnic system are both functional and structural^[13]. Thus, a reduced hepatic vascular cross-sectional area, owing to the formation of fibrosis and noduli, is well known^[13]. Swelling of hepatocytes and contraction of myofibrillary cells also contribute to hinder the hepatic outflow. Low content in endothelial cells of the vasodilator, nitric oxide (NO), may contribute to

Table 1 Haemodynamics in central and peripheral vascular beds in patients with cirrhosis

| Heart |
|--|
| Heart rate ↑ |
| Cardiac output ↑ |
| Left atrial volume ↑ |
| Left ventricular volume → (↑) |
| Left ventricular end-diastolic pressure → |
| Right atrial volume →↑↓ |
| Right atrial pressure →↑ |
| Right ventricular volume →↑↓ |
| Right ventricular end-diastolic pressure → |
| Systemic circulation |
| Plasma volume ↑ |
| Total blood volume ↑ |
| Central and arterial blood volume ↓ (→) |
| Noncentral blood volume ↑ |
| Arterial blood pressure ↓ (→) |
| Systemic vascular resistance ↓ |
| Arterial compliance ↑ |
| Total vascular compliance ↑ |
| Pulmonary circulation |
| Pulmonary blood flow ↑ |
| Pulmonary artery pressure →↑ |
| Pulmonary vascular resistance ↓ (↑) |
| Skeletal and cutaneous muscle circulation |
| Skeletal muscular blood flow ↑ → ↓ |
| Cutaneous blood flow ↑ → ↓ |
| Hepatic and splanchnic circulation |
| Hepatic blood flow ↑ → (↑) |
| Hepatic venous pressure gradient ↑ |
| Renal circulation |
| Renal blood flow ↓ |
| Glomerular filtration rate ↓ → |

↑, increase; →, no change; ↓, decrease. Parentheses denote minor changes.

hepatic vasoconstriction^[14]. In addition, vasodilators are produced in excess and escape degradation by the diseased liver, which may play a role in splanchnic and systemic vasodilatation in concert with local factors, especially shear stress^[6,15,16]. The result is an increased splanchnic inflow that aggravates and prolongs the portal hypertension, which is an essential element in cirrhosis^[7]. The formation of portosystemic collaterals with a highly increased flow through the azygos venous system contributes to the decreased overall vascular resistance^[5,7].

SYSTEMIC CIRCULATION

Overall systemic vascular resistance

Overall systemic vascular resistance is decreased in most patients with cirrhosis. Recent studies have shown that the circulation in most vascular territories is disturbed. A close look at the individual organs and tissues reveals vascular beds with decreased perfusion, normal perfusion, and increased perfusion. This indicates areas with high resistance (such as the kidneys), normal resistance (such as the brain), and low resistance (such as the splanchnic system)^[6,7] (Table 1). The pathogenesis of hyporeactivity of the vascular system in cirrhosis is multifactorial^[17]. Clinical and experimental observations favor the presence of a surplus of circulating vasodilators. Combined with

a decreased sensitivity to pressor substances, this leads to vasodilatation and reduced vascular resistance^[7,17]. Vasodilatation and the resulting activation of counter-regulatory mechanisms are closely related to the circulatory dysfunction in chronic liver disease^[18,19]. The reduced vascular responsiveness may be generated by a decrease in the number of receptors, a change in receptor affinity, and several postreceptor defects^[20]. Recent evidence suggests that all of these mechanisms are working in concert in cirrhosis^[20].

The coexistence of decreased splanchnic resistance (as in cirrhosis) and increased peripheral resistance (as in essential arterial hypertension) is likely and may depend on the altered circulating vasoactive substances, reactivity of vasomotor and regulatory systems, etc^[21,22]. The result may be increased, normal, or decreased systemic vascular resistance. However, most evidence indicates that a decreased overall systemic vascular resistance with a low arterial blood pressure is most often the outcome, even in patients with primary essential hypertension^[23-25]. Research on vascular hyporeactivity has primarily focused on calcitonin gene-related peptide (CGRP), NO, tumor necrosis factor- α (TNF- α), adrenomedullin, and endocannabinoids^[17,18]. In addition, there is substantial evidence of autonomic defects in patients with cirrhosis. Thus, the sympathetic nervous system is both dysfunctional and overactivated. The reason for vasoconstriction in some vascular areas is mainly overwhelming activity of pressor systems, but dysfunctional elements are also present^[5,19,26].

Arterial blood pressure

The arterial blood pressure depends on the cardiac output and the systemic vascular resistance. The former is primarily determined by heart rate, venous return, and myocardial contractility. The latter is determined by the tone of smooth muscle cells in the small arteries and arterioles, which is governed by complex local and central neurohumoral factors. Arterial blood pressure has a circadian rhythm, but is kept within its habitual range by negative feedback baroreceptor reflexes, volume regulation, and other regulatory systems^[12].

According to standardized recommendations, arterial blood pressure in patients with cirrhosis is often measured in the morning. However, 24-h determinations of blood pressure show that, during the day, the arterial blood pressure is substantially reduced compared with that of the controls, but at night it is normal^[27]. The shifted and flat blood pressure-heart rate relation in patients with cirrhosis suggests that the regulation of their blood pressure is abnormal^[27]. Moreover, the hemodynamic dysregulation is more pronounced with increasing severity of the liver disease^[9]. The abnormal diurnal variation of the arterial blood pressure and the drastic activation of the neurohormonal systems probably contribute to the sodium-water retention.

Studies of patients with cirrhosis and arterial hypertension show that the prevalence is substantially reduced with raised arterial blood pressure in only 3-7% of such patients^[21,24,28,29]. In addition, a few longitudinal studies have indicated that in patients with essential arterial hypertension, the raised arterial blood pressure is

Table 2 Haemodynamics and neuroendocrine mechanisms in patients with cirrhosis and different types of arterial hypertension

| | Cirrhosis | Cirrhosis with hypertension | Essential hypertension | Renovascular hypertension |
|--------------------------------------|-----------|-----------------------------|------------------------|---------------------------|
| Plasma and blood volume | ↑ | ↑ | → | → |
| Central blood volume | →↓ | →↓ | → (↑) | → (↑) |
| Systemic vascular resistance | ↓ | → | ↑ | ↑ |
| Arterial compliance | ↑ | → | ↓ | ↓ |
| Renal blood flow | ↓ | ↓→ | ↓ | ↓ |
| Sodium retention | ↑ | ↑ | →↑ | ↑ |
| Sympathetic nervous activity | ↑↑ | ↑ | → | →↑ |
| Renin-angiotensin-aldosterone system | ↑↑ | ↑→ | →↓ | ↑ |
| Vasopressin | ↑ | | → | → |

→ ↑ ↓, denote normal, increased, and decreased values, respectively; parentheses denote minor changes.

reduced to normal values after the onset of cirrhosis^[23]. Thus, patients with essential hypertension may become normotensive during the development of chronic liver disease. In patients with cirrhosis, the liver disease may protect against the development of arterial hypertension, and in patients with advanced cirrhosis raised arterial pressure is very uncommon^[24,30].

Gentilini *et al.* have investigated patients with early non-alcoholic cirrhosis and arterial hypertension^[21]. They found that the arterial hypertensive cirrhotic patients were normodynamic with a normal heart rate and cardiac output. By contrast, their normotensive cirrhotic counterparts were hyperdynamic in the supine position and normodynamic in the upright position. They concluded that hypertensive cirrhotic patients had an impaired cardiovascular response to postural changes, but a lesser degree of renal dysfunction while standing than that of their normotensive counterparts^[21]. No difference in the plasma renin activity of the normotensive and hypertensive cirrhotics was present^[21]. By contrast, Veglio *et al* had previously found that hypertensive cirrhotic patients had a lower plasma renin activity, both supine and upright^[29]. They concluded that the peripheral pressor effect of vasoactive hormones was increased and the effective blood volume was enhanced in arterial hypertensive cirrhotic patients^[29]. The authors of the present review have been unable to confirm Gentilini *et al's* result of a normodynamic circulation in early hypertensive cirrhotic patients and Veglio *et al's* hypothesis of an enhanced effective blood volume in these patients^[31]. However, Henriksen *et al's* study showed that hypertensive cirrhotic patients are less vasodilated than their normotensive counterparts^[31], (Figure 1). Essential differences in arterial hypertension and cirrhosis are summarized in Table 2.

Arterial compliance

Arterial compliance (i.e., an increase in intra-arterial volume relative to an increase in transmural arterial blood pressure) is raised in patients with decompensated cirrhosis^[9,21]. Recent investigations have shown that the altered static and dynamic characteristics of the wall

of large arteries in patients with cirrhosis are closely associated with the circulatory and homeostatic derangement^[9,21,32-35]. The changes in arterial mechanics are, at least in part, reversible^[32]. Arterial compliance is an important determinant of the coupling between the heart and the arterial system and of the dynamics of intravascular volume relocation^[36]. Reduced arterial blood volume and blood pressure are the most likely elements in the elevated arterial compliance in advanced cirrhosis^[9,13]. The increased arterial compliance in patients with cirrhosis is directly related to the severity of the liver disease and the circulating level of the vasodilator CGRP^[32]. It is inversely related to circulating adrenaline, but is not significantly related to the indicators of the potent vasoconstrictor systems (sympathetic nervous system (SNS) and endothelin-1)^[32-35]. In addition, the abnormal arterial compliance is related to blood volume abnormalities, hypoxia, and to the abnormalities in the C-type natriuretic peptide (CNP), but not to the atrial natriuretic peptide (ANP)^[33,35]. Arterial compliance is not affected by beta-adrenergic blockade, but infusion of the vasopressin analog terlipressin almost normalizes it^[37]. Both types of drugs are used in the treatment of portal hypertension^[38]. In arterial hypertensive cirrhotic patients, arterial compliance is normal or almost normal^[31].

Blood volume distribution

Patients with cirrhosis have increased blood and plasma volumes^[8,10]. Distribution of blood to the different vascular beds is abnormal and more so in severe liver disease^[39-42]. Moreover, dynamic, as well as static, methods have revealed that the central (thoracic) blood volume is most often decreased, (Figure 1c), whereas the non-central (especially splanchnic) blood volume is increased in patients with cirrhosis^[10,39]. The effective arterial blood volume is decreased, and the abnormal volume distribution contributes to the systemic circulatory derangement^[42]. Thus, the central circulation time (that is, central blood volume relative to the cardiac output) in cirrhosis has a significant relation to the survival and is substantially reduced also in cirrhotic patients with arterial hypertension^[31]. During volume expansion (either by blood transfusion, or infusion of albumin or osmotic material) most cirrhotic patients respond with a further reduction in systemic vascular resistance rather than an increase in arterial blood pressure^[33,42]. Future studies may disclose whether raised arterial blood pressure is indicative of normal arterial filling or the result of arterioconstrictive adaptation to a low arterial blood volume. Moreover, the balance between vascular areas with vasodilatation and vasoconstriction may be substantially altered in arterial hypertensive patients with cirrhosis^[31].

Cardiac dysfunction

In patients with advanced cirrhosis increased heart rate and stroke volume led to a high cardiac output^[18,36]. A left ventricular failure may be latent, because of the decreased afterload (as reflected by reduced systemic vascular resistance and increased arterial compliance)^[20,36]. Cardiac dysfunction may become manifest under strain or treatment with vasoconstrictors. Cirrhotic cardiomyopathy includes

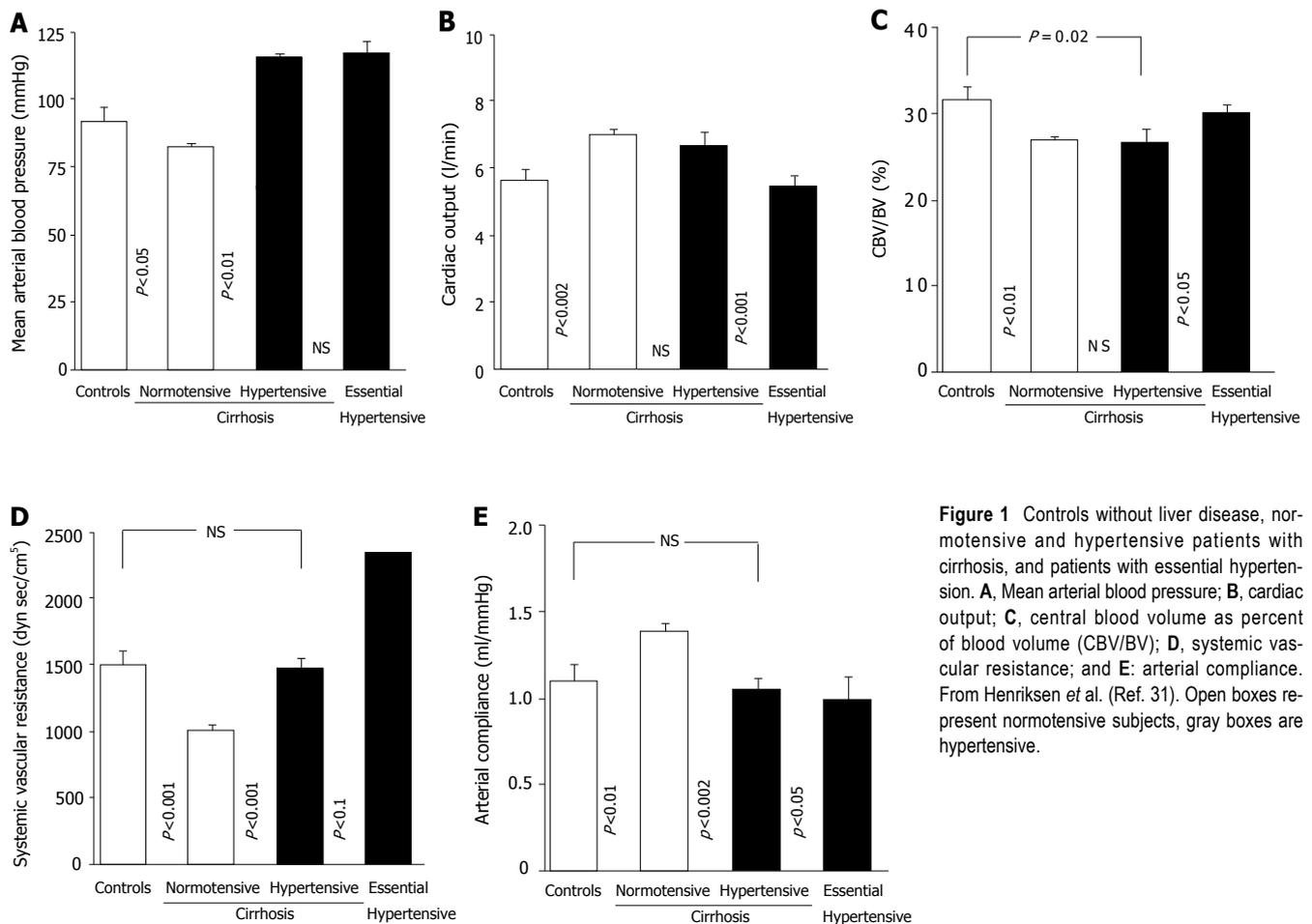


Figure 1 Controls without liver disease, normotensive and hypertensive patients with cirrhosis, and patients with essential hypertension. **A**, Mean arterial blood pressure; **B**, cardiac output; **C**, central blood volume as percent of blood volume (CBV/BV); **D**, systemic vascular resistance; and **E**: arterial compliance. From Henriksen *et al.* (Ref. 31). Open boxes represent normotensive subjects, gray boxes are hypertensive.

impaired cardiac contractility (systolic dysfunction), diastolic dysfunction, prolonged Q - T interval, reduced beta-adrenoceptor density, postreceptor signal defects, abnormal excitation-contraction coupling, and molecular abnormalities^[20,36,43]. Recently, it has been substantiated that the elevated levels of circulating pro-brain natriuretic peptide (pro-BNP) and brain natriuretic peptide (BNP) found in patients with cirrhosis are not related to the decreased disposal of these peptides, but to the increased production in the cirrhotic heart, thus indicating cardiac dysfunction in cirrhosis^[44]. In cirrhotic patients with arterial hypertension signs of myocardial involvement may be especially pronounced^[21,30].

Cirrhotic cardiomyopathy must be differentiated from alcoholic heart muscle disease, which is caused by impaired contractile protein synthesis and formation of immunogenic cardiac protein-acetaldehyde adducts. The presence of antibodies to these adducts may be a marker for alcoholic heart muscle disease and possibly for its pathogenesis^[36].

NEUROHUMORAL REGULATION OF ARTERIAL BLOOD PRESSURE AND VOLUME DISTRIBUTION IN CIRRHOSIS

Splanchnic arteriolar vasodilatation may lead to abnormal distribution of the circulating medium with a decrease in the effective blood volume and low arterial blood

volume and pressure, and to an expansion of the non-central blood volume, including the splanchnic bed^[14,26,42]. Most patients with advanced decompensated cirrhosis have a highly increased activity of vasopressor systems with a highly elevated plasma renin activity, circulating noradrenaline, and plasma vasopressin^[5,6,26]. In most cases there is a progressive increase from normal values to moderately increased values in preportal hypertensive patients to a further increase in portal hypertensive patients to highly increased values in ascitic patients, especially in those with functional renal failure^[5,45]. Recent studies indicate that infusion of albumin may suppress the renin-angiotensin-aldosterone system (RAAS), especially in patients with advanced cirrhosis, and this may prevent further circulatory dysfunction and even reduce it^[33].

In addition to the activation of the SNS, RAAS, and vasopressin, several studies have shown increased circulating endothelin-1, which indicates that endothelin systems are also activated in advanced cirrhosis^[46]. Systemic vasodilatation is a key feature in the activation of vasoconstrictive and sodium-water retaining systems^[5,16,22]. Systemic hemodynamic alterations are also important for the low renal blood flow and renal dysfunction in cirrhosis. The pressor systems most often counter-regulate the otherwise low arterial blood pressure in cirrhosis to a level almost within the normal range. Whereas a significant negative correlation of endothelin-1 to arterial blood pressure has been described, most studies have focused on the very low arterial blood pressure after the blockade

of the SNS (for instance, by clonidine, prazosin), RAAS (for instance by ACE-inhibitors, A II blockers), and the vasopressin effect (V_1 antagonists)^[16,45-48]. The activity in the pressor systems in advanced cirrhosis is among the most extreme known to pathophysiology. The presence of autonomic defects in patients with cirrhosis is evident from various studies on hemodynamic response to standardized cardiovascular reflex tests, such as heart rate variability, the Valsalva ratio, and isometric exercise^[17]. Most studies on these issues have found a high prevalence of autonomic dysfunction in cirrhosis directly associated with liver dysfunction and survival. The results point to receptor and postreceptor defects as important elements of the hyporeactive response in cirrhosis. Other studies suggest that the autonomic dysfunction secondary to liver dysfunction is temporary, and potentially reversible after liver transplantation^[48]. Arterial hypertensive cirrhotic patients may have less impaired blood pressure regulation and cardiovascular reflexes^[21], but this topic needs further investigation. In cirrhosis the plasma concentration of CGRP, a powerful vasodilating neurotransmitter, is increased^[18,32]. Circulating CGRP relates to cardiac output, systemic vascular resistance, arterial compliance, and liver dysfunction. In experimental studies, specific antagonists of CGRP partly reverse the vasodilatation and hyperdynamic circulation. Vallance and Moncada have proposed that NO could be implicated in the vasodilatation in cirrhosis^[49]. Several human and animal studies have supported this concept, whereas others have been unable to do so. To support the hypothesis, blockade of NO formation significantly improves the systemic hyperdynamic circulation, and infusion of the NO donor, L-arginine, aggravates the systemic vasodilatation and hyperdynamic circulation. Other vasodilating substances, such as adrenomedullin, the natriuretic peptides, and cannabinoids, may also be involved^[18]. Adrenomedullin, a polypeptide similar to CGRP, is increased in patients with decompensated cirrhosis. From the family of natriuretic peptides, ANP, BNP, CNP, urodilatin, the two cardiac peptides, ANP and BNP, may be raised in patients with cirrhosis^[35,45]. ANP is often increased in patients with ascites, owing to an enlarged left atrium and atrial stretching caused by a change in anatomical location of the heart. Circulating BNP (and pro-BNP) is especially increased in patients with cirrhotic cardiomyopathy^[36,44]. Urodilatin is often normal and CNP may be reduced in advanced cirrhosis^[35]. The increased natriuretic peptides and other vasodilators in cirrhosis may contribute to vasodilatation, and thereby counteract any increased arterial blood pressure from the coexistence of essential or secondary arterial hypertension.

According to the peripheral arterial vasodilatation theory of Schrier and co-workers, peripheral and splanchnic vasodilatation leads to a reduction in systemic vascular resistance and arterial blood volume and pressure, which are primary events in the retention of sodium and water^[6]. Over the past few years several vasodilators have been candidates, and those mentioned above are involved in the cirrhotic vasodilatation, but further research is needed in order to answer a number of unsolved questions, especially in relation to the vascular beds which are dilated and constricted.

KIDNEY FUNCTION AND FLUID RETENTION

Functional renal failure in cirrhosis (hepatorenal syndrome) is characterized by renal insufficiency consequent on hepatic failure^[5,16,45,47]. No clinical or patho-anatomical signs of other known causes of renal failure are present. In general, the hepatorenal syndrome is characterized by a decrease in the renal blood flow and glomerular filtration rate, avid sodium-water retention, azotemia, and later oliguria. The condition may be considered as a progressive functional nephropathy, consequent on dyscirculatory and dysregulatory collapse^[5,16]. The renal perfusion pressure is decreased, not only because of the low arterial blood pressure, but also because of the elevated renal venous pressure, especially in patients with tense ascites^[16]. The enhanced sympathetic nervous activity may alter the autoregulation curve of the kidney with a shift to the right^[50]. Patients with hepatorenal syndrome have the highest plasma levels of circulating catecholamines, and there are several indicators that enhanced renal SNS activity plays an important role in the renal vasoconstriction. The highly significant inverse relation between renal noradrenaline overflow on the one hand and renal blood flow on the other may illustrate this^[16].

Activation of the RAAS may contribute to decreased renal perfusion, but the RAAS may also have more complex regulatory effects inside the kidney^[16,18,26]. It has been shown that vasopressin does not substantially change renal perfusion^[26]. Noradrenaline, adrenaline, and endothelin-1 are important elements in the renal hypoperfusion and sodium-water retention in advanced cirrhosis^[16,46,47]. Local vasodilators, such as prostaglandins and NO, most likely compensate for, at least in part, the progressive renal vasoconstriction seen in advanced cirrhosis^[5]. In decompensated patients, normalization of or an increase in arterial blood pressure may increase renal perfusion and improve renal function^[50]. Accordingly, a combination of prolonged administration of ornisipressin or terlipressin and albumin has recently been reported to improve functional renal failure in cirrhosis^[45]. Treatment with alpha-adrenergic blockers and potentially with endothelin-1 blockers may reverse renal vasoconstriction, but their lowering effect on arterial blood pressure may overrule beneficial local effects on the kidney^[16]. Improvement of the cardiac dysfunction in cirrhosis may also improve the renal dysfunction^[51].

Loyke found that renal and neurogenic mechanisms able to elevate the blood pressure remained intact in patients with cirrhosis^[25,28]. This conclusion was based on several patients with coexistent cirrhosis and glomerular nephritis. However, today this is a rather uncommon combination, at least in patients with alcoholic liver disease. Later, Loyke reported examples of renal hypertension, which returned to normotension when the patients developed cirrhosis^[23].

The hemodynamic changes in cirrhosis point towards renal hypoperfusion as being, at least initially, a physiological response to the changes in the systemic circulation. Increased SNS activity is a pathogenic factor, but other systems, such as the RAAS and endothelins, may also play a role. Angiotensin II mainly acts on the efferent arteriole and a low dose of an ACE-inhibitor

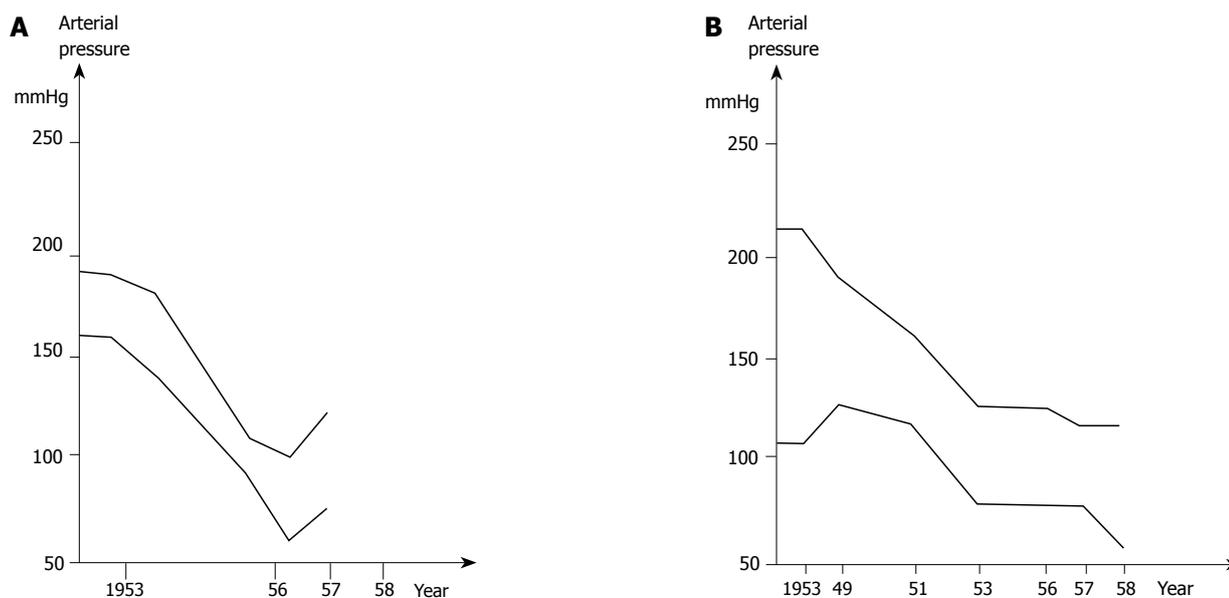


Figure 2 Change in arterial blood pressure in two patients with arterial hypertension. (A, 39-year-old woman with essential hypertension; B, 59-year-old woman with hypertension of renal origin.) A decrease in the arterial blood pressure is seen when the patients develop cirrhosis. From Loyke 1962 (reproduced with the permission of Archives of Internal Medicine, Ref. 23).

may induce a significant reduction in glomerular filtration and a further reduction in sodium excretion, even in the absence of a change in arterial blood pressure. This suggests that the integrity of the RAAS is important for the maintenance of renal function in cirrhotic patients, and that RAAS overactivity does not solely contribute to the adverse renal vasoconstriction. Infusion of terlipressin or ornipressin has, along with a rise in arterial blood pressure, demonstrate beneficial effects with increased GFR in patients with hepatorenal syndrome.

A few cases have been reported where patients with cirrhosis with renal involvement may develop arterial hypertension, especially in patients with early cirrhosis^[52]. After the onset of severe cirrhosis most patients with renal hypertension become normotensive, like patients with essential hypertension (Figure 2). However, nephropathy in cirrhosis most often leads to low arterial blood pressure, in spite of the highly increased activity in the RAAS system. In most types of bilateral organic renal failure with vascular involvement and sodium retention, hypertension will be the outcome, but not in patients with cirrhosis, where the mechanisms are quite the reverse. Alterations in hemodynamic and homeostatic mechanisms in renal hypertension and cirrhosis are summarized in Table 1.

ARTERIAL HYPERTENSION IN CIRRHOSIS

The prevalence of arterial hypertension in cirrhotic patients is substantially reduced, especially in advanced cirrhosis. Characteristic findings in patients with cirrhosis are vasodilatation with low overall systemic vascular resistance, high arterial compliance, increased cardiac output, secondary activation of counter-regulatory systems (RAAS, sympathetic nervous system, release of vasopressin), and resistance to vasopressors. The vasodilatory state is mediated through adrenomedullin, CGRP, NO, and other va-

sodilators, and is most pronounced in the splanchnic area. This constitutes an effective (although relative) counterbalance to raised arterial blood pressure. Subjects with arterial hypertension may become normotensive during the development of chronic liver disease, and arterial hypertension is rarely manifested in patients with cirrhosis, even in cases with renovascular disease and high circulating renin activity. There is much dispute as to the understanding of homeostatic regulation in cirrhotic patients with manifest arterial hypertension. This condition most likely includes the combination of vasodilatation and vasoconstriction in parallel. As hypertensive patients are often effectively treated with diuretics, calcium channel antagonists, beta-blockers, ACE-inhibitors, etc., and some of these drugs are also applied in the treatment of cirrhosis and portal hypertension (Table 3), the natural history and prevalence of cirrhosis in patients with arterial hypertension, arterial hypertension in patients with cirrhosis, and the inter-relationship of these two diseases may be difficult to study today in prospective and untreated cases. Nevertheless, such studies are relevant, since there are many unsolved questions.

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Table 3 Effects of propranolol, diuretics, terlipressin, and albumin on pressures, vascular resistance, vascular compliance, blood flow, blood volume distribution, portal pressure, and sodium excretion in patients with cirrhosis

| | Non-selective beta-blocker | Diuretics | Terlipressin | Albumin |
|-------------------------------------|-------------------------------|-----------|--------------|---------|
| Arterial blood pressure | (↓) | (↓) | ↑ | → |
| Systemic vascular resistance | ↑↑ | | ↑↑ | ↓ |
| Arterial compliance | → | → | ↓ | ↑→ |
| Total effective vascular compliance | | ↓→ | | |
| Cardiac output | ↓↓ | ↓ | ↓ | ↑ |
| Central and arterial blood volume | → | (↓) | ↑ | ↑→ |
| Plasma volume | → | ↓ | ↓ | ↑ |
| Hepatic venous pressure gradient | ↓→ | ↓ | ↓ | → |
| Urinary sodium excretion | → | ↑ | ↑ | ↑ |

→ ↑ ↓, denote normal, increased, and decreased values, respectively. Parentheses denote minor changes.

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GASTRIC CANCER

Construction of retroviral vector of p^{125FAK} specific ribozyme genes and its effects on BGC-823 cells

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Abstract

AIM: To construct the retroviral vector of p^{125FAK} specific ribozyme genes and to explore the feasibility of ribozyme in BGC-823 gene therapy *in vitro*.

METHODS: A hammerhead ribozyme DNA targeting p^{125FAK} mRNA from nt 1010 to nt 1032 was synthesized and recombined into the retroviral vector pLXSN forming pLRZXSXN recon. Using the lipofectin-mediated DNA transfection technique, pLRZXSXN was introduced into BGC-823 cells. The effects of ribozyme on the growth of BGC-823 cells and apoptosis were studied by cell colony assay, flow cytometry (FCM), reverse transcriptase-polymerase chain reaction (RT-PCR), detection of DNA fragmentation and electron microscopy.

RESULTS: The number of BGC-823 cell colonies was inhibited by 56% after the cells were treated for 48 h. The cell proliferation was inhibited effectively by p^{125FAK} ribozyme and the inhibitory effect depended on the concentration and the time of incubation. The expression of p^{125FAK} mRNA and protein P¹²⁵ decreased sharply in BGC-823 cells treated with p^{125FAK} ribozyme. The characteristics of apoptosis, namely sub-G1 peak, DNA fragmentation and morphological changes, were revealed in BGC-823 cells treated with p^{125FAK} ribozyme.

CONCLUSION: p^{125FAK} ribozyme decreases p^{125FAK} gene expression and induces apoptosis of human gastric cancer cells *in vitro*.

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Key words: Ribozyme; p^{125FAK} gene; Stomach neoplasm; Apoptosis

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INTRODUCTION

p^{125FAK} is a non-receptor cytoplasm protein tyrosine kinase (PTK) with a molecular weight of 125 ku, which has unique structural and functional characteristics. Focal adhesion kinase (FAK) regulates multiple cellular processes including growth, differentiation, adhesion, motility and apoptosis^[1-4]. The high expression of p^{125FAK} is possibly a part of cell incidents contributing to the invasion and metastasis of tumors^[5-7]. Ribozyme (RZ) is defined as a kind of small RNA molecule that has catalytic activity and can inhibit the translation process of mRNA after RZ combines with the complementary sequences of mRNA and incises it^[8-11]. In this study, a hammerhead ribozyme DNA targeting p^{125FAK} mRNA from nt 1010 to nt 1032 was synthesized according to p^{125FAK} cDNA sequences, and recombined into the retroviral vector pLXSN forming pLRZXSXN recon. Using the lipofectin-mediated DNA transfection technique, pLRZXSXN was introduced into BGC-823 cells to study the expression of RZ and its effect on BGC-823 cells.

MATERIALS AND METHODS

Materials

BGC-823 cells were purchased from Shanghai Cytobiology Institute of Chinese Academy of Medical Sciences. *E. coli* DH-5 were donated by Hematopathy Laboratory of Fujian Medical University. Restriction enzymes (HindIII, pst I, BamH I, Xho I) and T₄DNA ligase, X-gal, IPTG were all from Promega. pBluescript@SK plasmid was from Stratagene. RT-PCR kit was from Promega, LipofectinTM was from Gibco/BRL, p^{125FAK} (H-1) was from American Santa Cruz. S-P kit and DAB kit were from Maxim Biotechnology and Zymed Lab Inc, respectively.

Design and synthesis of RZ template

GUU triplets of nt 1010 to nt 1032 in p^{125FAK} cDNA base sequences were used as cleavage sites. Two small nucleotide sequences complementary to the cleavage

Table 1 Primer sequence and fragment length

| Primer | Sequence | Fragment length (bp) | Annealing Temp (°C) |
|---------------------|------------------------------|----------------------|---------------------|
| p ^{125FAK} | A: 5'TTCTTCTATCAACAGGTGAAG3' | 632 | 55 |
| | B: 5'CTGCGAGGTTCCATTACCAG3' | | |
| β-actin | A: 5'GGCATGGGTCAGAAGGATTCC3' | 500 | 55 |
| | B: 5'ATGTCACGCACGATTCCCGC3' | | |

Note: A and B represent the sense and anti-sense primers, respectively.

sites of target RNA were put at the two ends of the conservative core sequence-(the hammerhead structure) to form a typical active incisive secondary structure of RZ as previously described^[12]. The two limbs and complementary ribonucleotides of target RNA were all 10 nt. RZ gene consisted of 2 completely complementary trains called trains A and B, consisting of 60 bases respectively. Two cleavage sites and 3 protective bases were synthesized by Shanghai Sangon Technology Corporation. The sequences of RZ gene were 5'CGGACTCATCAGCAAGCTGGAT AAGCTTCGT-3' for train A and 3'GCCTGAGTAGTCG TTCGACCTATTCGAAGCA-5' for train B.

Construction of sequencing vector and DNA sequence analysis

Plasmid extraction, restrictive enzymolysis reaction, ligase coupled reaction, preparation of competent germ, *E coli* transfection, agarose gel electrophoresis and DNA fragment retrieval were routinely carried out. DNA sequencing was undertaken by Shanghai Sangon Technology Corporation.

Construction of pLRZSN recon

Sequencing of a fragment of 81 bp was carried out with Bam HI and Xho I, retrieved by polyacrylamide gel electrophoresis(PGE), recombined into pLXSN cleavage sites of Bam HI and Xho I and then labeled as pLRZSN. The pLRZSN from QIAGEN was exactly quantified and sub-packaged for later use.

Transfection of BGC-823 cells

BGC-823 cells in log growth phase were divided into BGC 823 cell blank group, BGC-823 cells + LipofectinTM group, BGC-823 cells + pLXSN group and BGC-823 cells + pLRZSN group. Liguor A was obtained by adding 4 μg DNA (pLXSN or pLRZSN) to 100 μL serum-free RPMI1640, while liguor B was obtained by adding 10μL LipofectinTM to 90 μL serum-free RPMI1 640. Liguors A and B were mixed after 30 min and stood for 15 min. At the same time, 1×10⁶ BGC-823 cells in log growth phase were washed. After resuspension in 0.8mL serum-free RPMI1640, the mixture of liquors A and B was added into the suspension and incubated at 37 °C in an atmosphere containing 5% CO₂ for 6 h . Four mL RPMI1640 containing 10% serum was added into the suspension and incubated for 48-72 h and then the BGC823 cells were collected.

Colony forming experiment

After incubated for 48 h, the 4 groups of cells were digest-

ed in monoplast suspensions and inoculated into 24-well plates (200/well). At the same time, 3 parallel wells were being designed. After being incubation for 7 d, colonies formed when the number of cells was over 50. The number of colonies was calculated under microscope and the colony forming efficiency was determined.

Detection of p^{125FAK} protein

After being incubated for 48 h, the 4 groups of cells were digested in monoplast suspensions and inoculated onto slides. When the slides were open-air dried and fixed in cold acetone for 10 min, immunocytochemistry dyeing was carried out by S-P method. Positive cell cytoplasm was buffy, nuclei and negative cells were not stained. p^{125FAK} positive cell labeling index (PI = p^{125FAK} positive cell number under one field of vision/1000×100%) was calculated. After being incubated for 48 h, the 4 groups of cells were digested in monoplast suspensions and prepared into specimens. p^{125FAK} protein was detected by flow cytometry (Bio-Rad, Brite-HS).

Evaluation of p^{125FAK} mRNA

Total RNA was extracted from the cells after incubation for 48 h. cDNA synthesis and PCR amplification were performed as previously described^[13]. p^{125FAK} gene nucleotide sequence to be amplified the primers used are listed in Table 1.

Detection of apoptosis of BGC-823 cells

After being incubated for 48 h, the 4 groups of cells were digested in monoplast suspensions, poached with PBS, centrifuged and incubated for 30 min in Kenesis50 kit (BioRad) and the DNA contents were analyzed by FCM. The ultra-structure of BGC-823 cells was observed under transmission electron microscope. The 4 groups of cells incubated for 48 h were prepared and photographed. Extraction and electrophoresis of DNA apoptotic fragments were carried out following the directions of apoptotic DNA ladder kit (Roche Corporation).

Statistical analysis

The data were expressed as mean ± SD. The variance analysis and significant difference test were carried out by SPSS 10.0.

RESULTS

Effect of RZ on colony forming efficiency (CFE) of BGC-823 cells

The results of colony forming experiment are summarized in Table 2. RZ could inhibit colony formation of BGC-823 cells ($P < 0.05$).

Effect of RZ on expression of BGC-823 p^{125FAK} protein

After the cells were incubated for 48 h, the expression rate of p^{125FAK} protein was 88.4% in control group, 79.55% in liposome group, 77.08% in kenovector group, and 46.09% in ribozyme group. A significant difference was found among the 4 groups. The positive number of BGC823 cells expressed in the p^{125FAK} protein decreased sharply and the intensity of expression also weakened. PI was obviously smaller in RZ group than in other three groups

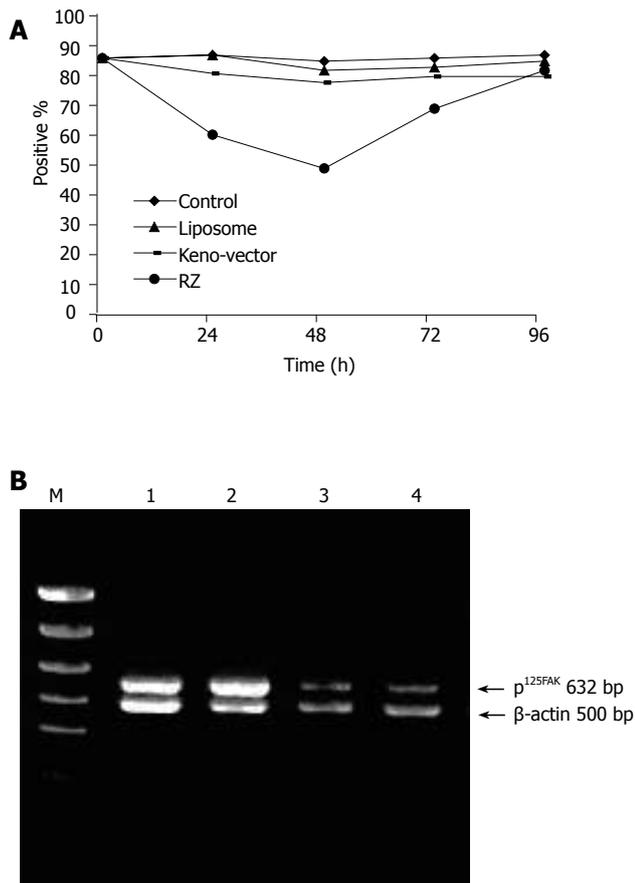


Figure 1 Expression of p^{125FAK} protein (A) and mRNA (B) in BGC 823 cells M: Marker; lane 1: control; lane 2: liposome; lane 3: keno-vector; lane 4: RZ

Table 2 Effect of RZ on colony formation of BGC-823 cells

| Groups | CFE (%) | CIE (%) |
|------------------|------------------------|---------|
| Control group | 51.24±6.8 ^a | |
| Liposome group | 46.43±8.6 ^a | 9.38 |
| Kenovector group | 45.32±5.2 ^a | 11.55 |
| Ribozyme group | 16.14±3.5 ^b | 68.50 |

^a*P*<0.05 vs other groups.

(Figure 1A).

Effect of RZ on p^{125FAK} mRNA expression in BGC 823 cells

PCR product electrophoresis showed 632 bp and 500 bp bands. The fluorescence intensity value of p^{125FAK} and β-actin was 1.23 in control group, 0.98 in liposome group, 0.92 in keno-vector group, and 0.38 in RZ group, indicating that the level of p^{125FAK} mRNA in BGC-823 cells decreased sharply than that in other three groups (Figure 1B).

Apoptosis of BGC-823 cells induced by RZ

Apoptotic peak (sub-diploid peak) appeared in RZ group but not in other three groups (Figure 2).

Karyopynosis, chromatin margination, complete caryotheca, condensed cytoplasm with deep staining were found in RZ group. Larger karyoplasmic ratio, puffed

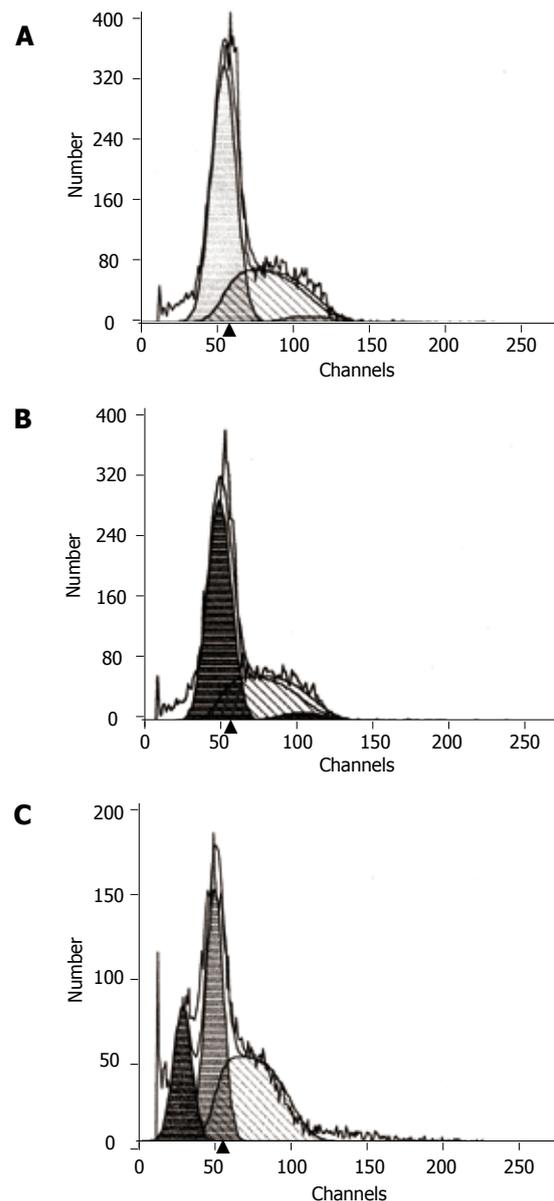


Figure 2 Cell cycle analysis of cells cultivated for 48 h in liposome group (A), keno-vector group (B) and RZ group (C).

chromatin, clear plasmosome, and abundant cytoplasm were seen in other three groups. These cells were poorly-differentiated BGC-823 cells which did not undergo apoptosis (Figure 3).

Typical DNA fragments were seen in RZ group but not in other three groups (Figure 4).

DISCUSSION

Recent studies showed that when malignant cells adhere to extra-cellular matrix (ECM), p^{125FAK} tyrosine self phosphorylation takes place and its activity increases^[14-16]. The precise mechanism of p^{125FAK} underlying apoptosis is not clear. But when malignant cells spread or migrate, p^{125FAK} regulates the formation of adhesion plaque or takes part in signal cascade conduction and inhibits apoptosis by informing karyoplasts to that cells are ECM-anchored^[17,18]. Anti-sense oligonucleotides of p^{125FAK} have been used to

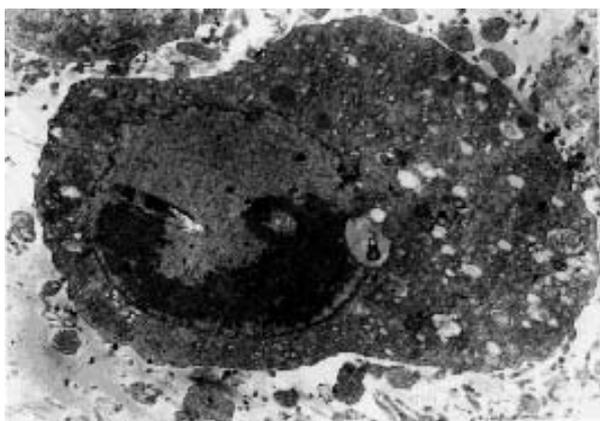


Figure 3 Apoptotic changes of BGC-823 cells in RZ group.

inhibit the expression of p^{125FAK} gene in order to induce apoptosis of malignant cells^[19,20], but the result is not satisfactory because the number of target RNAs is so larger that complete blocking is impossible and anti-sense oligonucleotides are so easy to be degraded. RZ may solve the above problems. Ribozyme is a small RNA molecule that has catalytic activity. On the one hand, it can combine with complementary sequences of mRNA to block the translation of mRNA. moreover, it can incise mRNA and promote the degradation of mRNA^[10,21]. Symons^[8] reported that RZ can be used in treatment of viral disease and tumor. RZ genes such as c-erbB-2, P53, Ras, TGF- β , can be used in oncotherapy^[22-25]. Qian *et al.*^[26] reported that FGFR3 RZ gene could inhibit the growth of myeloma cells and promote their apoptosis. But studies on p^{125FAK} RZ gene are relatively fewer. In the present study, a hammerhead ribozyme DNA targeting p^{125FAK} mRNA from nt 1010 to nt 1032 was synthesized according to p^{125FAK} cDNA sequences, and recombined into the retroviral vector pLXSN forming pLRZXSXN recon. Using the lipofectin-mediated DNA transfection technique, pLRZXSXN was introduced into BGC-823 cells to inhibit the growth of BGC-823 cells to observe the typical changes of apoptosis under electron microscope. The results demonstrate that p^{125FAK} RZ gene is sequence specific and can be used in treatment of gastric carcinoma.

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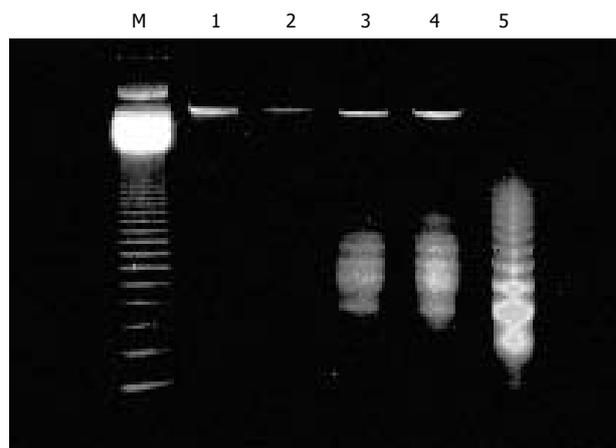


Figure 4 DNA fragments of BGC-823 cells in different groups M: 123 bp DNA ladder; lane1: liposome group; lane 2: keno-vector group; lane 3: RZ group for 24 h; lane 4: RZ group for 48 h; lane 5: RZ group for 72 h.

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Down-expression of tumor protein p53-induced nuclear protein 1 in human gastric cancer

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CONCLUSION: TP53INP1-positive rate decreases with the progression of gastric cancer. TP53INP1 protein negativity is significantly associated with aggressive pathological phenotypes of gastric cancer. TP53INP1 is related to the apoptosis of gastric cancer cells. The decreased expression of the TP53INP1 protein may reflect the malignant grade of gastric cancer and is regarded as an adverse prognostic factor.

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Key words: Tumor protein 53-induced nuclear protein 1; p53; Gastric cancer

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Abstract

AIM: Overexpression of tumor protein p53-induced nuclear protein 1 (TP53INP1) induces G₁ cell cycle arrest and increases p53-mediated apoptosis. To clarify the clinical importance of TP53INP1, we analyzed TP53INP1 and p53 expression in gastric cancer.

METHODS: TP53INP1 and p53 expression were examined using immunohistochemistry in 142 cases of gastric cancer. The apoptosis of gastric cancer cells was analyzed using the TUNEL method. The relationship between the expression of TP53INP1 and clinicopathological factors was statistically analyzed.

RESULTS: TP53INP1 was expressed in 98% (139/142 cases) of non-cancerous gastric tissues and was down-expressed in 64% (91/142 cases) of gastric cancer lesions from the same patients. TP53INP1 expression was significantly decreased (43.9%) in poorly differentiated adenocarcinoma compared with well or moderately differentiated adenocarcinoma (81.6%). Cancers invading the submucosa or deeper showed lower positivity (59.1%) compared with mucosal cancers (85.2%). Decrease or loss of TP53INP1 expression was significantly correlated with lymphatic invasion (54.3% vs 82.0% without lymphatic invasion) and node-positive patients (31.3% vs 68.3% in node-negative patients). P53 was expressed in 68 (47.9%) patients of gastric cancer, whereas it was absent in normal gastric tissues. A significant association was also observed between TP53INP1 status and the level of apoptosis in tumor cells: the apoptotic index in TP53INP1-positive tissues was significantly higher than that in TP53INP1-negative portions. Finally, when survival data were analyzed, loss of TP53INP1 expression had a significant effect in predicting a poor prognosis ($P=0.0006$).

INTRODUCTION

Tumor protein 53-induced nuclear protein 1 (TP53INP1) is a p53-inducible gene encoding two protein isoforms able to modulate p53 biological activities^[1-4]. TP53INP1 expression is strongly induced *in vivo* in mice with acute pancreatitis^[1], and *in vitro* in several cell lines submitted to various stress agents^[2,4]. Over-expression of TP53INP1 induces cell cycle arrest in G₁ phase and enhances the p53-mediated apoptosis^[3]. TP53INP1 co-localizes with p53 and the serine-threonine p53-kinase HIPK2^[5] within the promyelocytic leukemia protein nuclear bodies (PML-NBs) and physically interacts with these proteins modifying the p53 transcriptional activity on several p53 target genes^[3]. TP53INP1 thus appears as a key-element in p53-mediated cell death and cell cycle arrest, induced by cellular stresses. The multi-step model of carcinogenesis in gastric cancer, the second most common cancer leading to death in the world, suggests accumulation of genetic alterations, epigenetic changes and posttranslational modifications. It often metastasizes to other organs, including the liver, lung, and ovary^[6]. Multiple factors are known to be related to gastric carcinogenesis, including Epstein-Barr virus^[7] and *H. pylori* infections^[8], microsatellite instability^[9]. From the molecular point of view, it has now been established that gastric carcinogenesis is involved the accumulation

of mutations in oncogenes and tumor suppressor genes controlling epithelial cell growth and differentiation^[10-14]. In particular, TP53 mutations are frequently seen in gastric cancers and correlates with gastric cancer prognosis^[15,16]. However, the molecular alterations and their role in gastric cancer still remain to be fully defined.

Previous works implied that *TP53INP1* is a pro-apoptotic gene induced by p53^[2]. Overexpression of TP53INP1 promotes G1 arrest and apoptosis through the p53-mediated pathway^[3]. The aim of the present study was to analyze the expression patterns of TP53INP1 in a large series of gastric carcinomas to (1) identify the possible modulation of TP53INP1 expression; (2) investigate the association with apoptotic activity; (3) analyze the relationship with clinicopathologic parameters, and evaluate its prognostic value.

MATERIALS AND METHODS

Patients and specimens

One hundred and forty-two patients with gastric cancer were enrolled in this study. The areas adjacent to cancer lesions were used as non-malignant gastric tissue. The patients underwent operation at the Cancer Research Institute Hospital, Kanazawa University. The histological classification was defined using the Japanese classification of gastric carcinoma^[17]. Intestinal type was defined as either papillary or well to moderately differentiated tubular adenocarcinoma. Diffuse type was defined as poorly differentiated adenocarcinoma, signet-ring cell carcinoma, or mucinous adenocarcinoma. The series included 104 men and 38 women, and the mean age was 63.1 ± 10.6 years. There were 76 and 66 cases of differentiated and undifferentiated type, respectively.

Immunohistochemistry

A standard avidin-biotin-peroxidase complex method (ABC) was used for immunostaining. Deparaffinized sections were treated by microwaving at a high power for 5 min twice in a 10 mmol/L citrate buffer to retrieve antigenicity. After washing with PBS, the sections were immersed in 3% hydrogen peroxide in methanol for 20 min to block any endogenous peroxidase activity. Then the ABC staining system kit (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was used for the detection. Sections were incubated with 10% normal serum for 1 h to inhibit nonspecific antibody binding. Then, sections were incubated overnight at 4 °C with 6 µg/mL of rat anti-human monoclonal antibody raised against *TP53INP1* (kindly provided by Carrier). After washing with PBS, detection was done by successively incubating the sections with biotinylated goat anti-rat IgG for 30 min, and avidin-biotin-HRP for 30 min. After extensive washings with PBS, sections were stained with 3,3'-diaminobenzidine for 2-10 min. Then, sections were counterstained with hematoxylin, dehydrated, and mounted. Nuclei were lightly counterstained with Mayer's hematoxylin. TP53INP1-positive cells were counted in fields chosen at random (100 × magnification), and the percentage of the number of positive cells per 1 000 cells was expressed as TP53INP1-positive index (%). Using the same method we counted the

TP53INP1-positive in nucleus and in cytoplasm under the microscopy with a 200× magnification. The normal IgG was used as a negative control.

Immunohistochemistry for p53 was performed using a DAKO LSAB kit (DakoCytomation, Kyoto, Japan). The primary antibody was mouse monoclonal antibody against human p53 (DO-7, Nichirei Inc., Tokyo, Japan). The procedure was according to the protocol from the company. Finally the sections were incubated with DAB substrate as a chromogen. The cell nuclei were also lightly counterstained with Mayer's hematoxylin.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)

TUNEL-positive epithelial cells were detected in the sections using ApopTag Plus peroxides *in situ* apoptosis detection kit (Chemicon International, Inc., Temecula, CA, USA). Briefly, after pretreatment with 20 µg/mL of proteinase K and 3% hydrogen peroxide, sections were incubated with a labeling mixture for 1 h at 37 °C. Then 55 µL of anti-digoxigenin-peroxidase was deposited on the sections and incubated for 30 min. The reaction products were visualized by 3,3'-diaminobenzidine substrate. Nuclei were counterstained with methyl green for 10 min. After washing with *n*-butanol, the sections were dehydrated, and mounted. Apoptotic index (%) corresponding to the number of labeled nuclei per 1 000 nuclei was calculated.

Statistical analysis

Experimental results were expressed as mean ± SE. Difference between the means was evaluated by the Mann-Whitney *U* test. $P < 0.05$ was considered statistically significant. The statistical analysis in this paper such as Kaplan-Meier analysis and Cox regression model was performed by using the software of StatView-5.0 Macintosh (Tokyo, Japan).

RESULTS

TP53INP1 was expressed in non-malignant gastric tissues and its expression was reduced in gastric cancer tissues

In the non-neoplastic gastric mucosa, TP53INP1 was mainly located in the cytoplasm of epithelial cells (Figures 1A and 1B). Some nuclei were also stained for TP53INP1 (Figure 2). Similar patterns were observed for intestinal metaplasia samples (Figure 1A). To determine if TP53INP1 is differentially expressed in gastric carcinomas or if it is stage-related, we did immunohistochemical analysis on 142 samples (76 cases of intestinal type, and 66 cases of diffuse type). All the cancer samples had accompanying non-malignant tissues, 98% of them were positive for TP53INP1 expression (Table 1) and thus could be used as internal control. In contrast, the expression of TP53INP1 protein was seen in 91 cases (64%), the other 51 samples (36%) were TP53INP1-negative (Figure 1B, arrow). Overall, TP53INP1 expression in gastric cancers was significantly lower both in cell cytoplasm and nucleus than in non-malignant gastric tissue ($P < 0.0001$, Table 1 and Figure 2). However, the expression of TP53INP1 was decreased in well-differentiated tubular adenocarcinoma (Figure 1C), and was markedly diminished in poorly

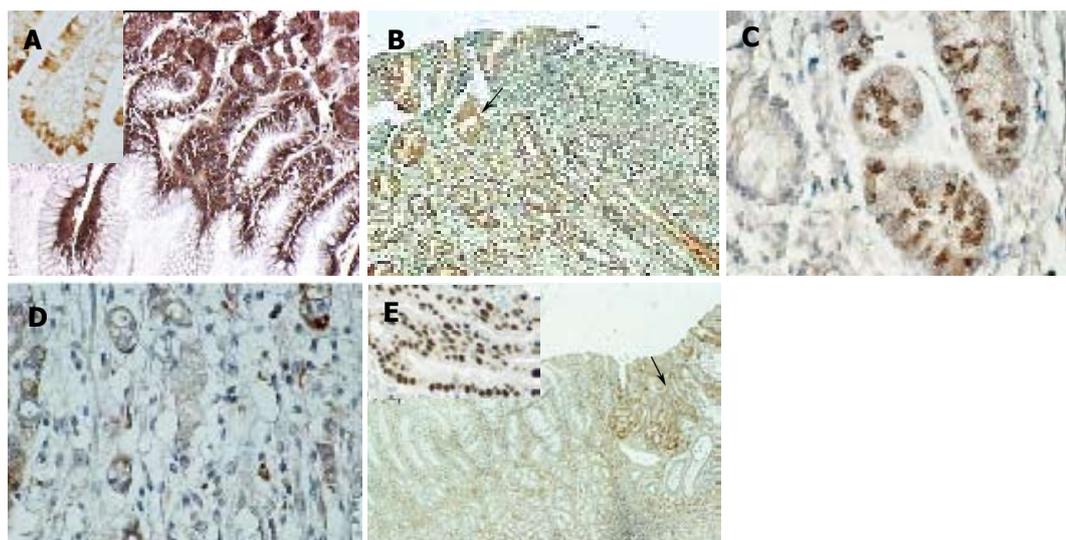


Figure 1 Immunohistochemical analysis of TP53INP1 expression in gastric carcinoma. **A:** TP53INP1 was strongly expressed in normal gastric mucosa or intestinal metaplasia foci (inset); **B:** TP53INP1 expression decreased in gastric carcinoma (arrow); **C:** well differentiated tubular carcinoma exhibited moderate alteration of TP53INP1 expression; **D:** poorly differentiated carcinoma showed weak staining with TP53INP1 antibody; **E:** P53 expression increased in gastric carcinoma (arrow).

Table 1 TP53INP1 expression in gastric cancer *n* (%)

| | | Non-malignant gastric tissue (<i>n</i> = 142) | Gastric cancer (<i>n</i> = 142) ¹ | <i>P</i> value |
|----------|----------|--|---|----------------|
| TP53INP1 | Positive | 139 (98) | 91 (64) | <0.0001 |
| | Negative | 3 (2) | 51 (36) | |
| P53 | Positive | 0 (0) | 68 (47.9) | <0.0001 |
| | Negative | 142 (100) | 74 (52.1) | |

¹In gastric cancer the *P* value between TP53INP1 and P53 was 0.006.

differentiated-type cancer (Figure 1D).

We next examined whether TP53INP1 expression is associated or not with the development and progression of gastric carcinoma. The clinical details of the cohort of patients and the statistical analysis are listed in Table 2. Only two non significant associations were observed, i.e., age and gender. TP53INP1 negativity was associated with gastric body and antrum tumor location ($P=0.0193$), with poorly differentiated adenocarcinoma (diffuse type) ($P<0.0001$). With regard to the depth of invasion, the positive TP53INP1 expression rate was 100% in intramucosal tumors (5/5), 81.8% when mucosa was invaded (18/22), 76.5% in muscularis propria (26/34), 54.3% in subserosa (25/46), and 48.6% in serosa (17/35). These results showed that alteration of TP53INP1 expression was correlated to the staging of the tumors. The difference was statistically significant when T1 tumors were compared to the other stages ($P=0.0111$, Table 2). In addition, TP53INP1 was significantly expressed in node-negative patients ($P=0.0037$), and significantly associated with lymphatic invasion-negative patients ($P=0.0010$). Taken together, these results indicated that loss of TP53INP1 expression was significantly associated with poorly differentiated histology, deep invasion, lymph node invasion, and metastasis.

TP53INP1 and apoptosis

TP53INP1 modulates the cell cycle arrest and programmed cell death^[3]. To investigate whether the modulation of TP53INP1 expression is associated with differences in apoptotic activity, TUNEL assays were done in all the

Table 2 Correlation between TP53INP1 expression levels and clinicopathologic features in gastric cancer

| | TP53INP1-positive (<i>n</i> = 91 of 142 patients) | TP53INP1-negative (<i>n</i> = 51 of 142 patients) | <i>P</i> value |
|-------------------------------|--|--|----------------|
| Age (years) | | | |
| <60 | 31 | 18 | NS |
| ≥60 | 60 | 33 | |
| Sex | | | |
| Male | 68 | 36 | NS |
| Female | 23 | 15 | |
| Location | | | |
| Cardia | 20 | 22 | 0.0193 |
| Body | 58 | 26 | |
| Antrum | 13 | 3 | |
| Histological type | | | |
| Differentiated ^a | 62 | 14 | <0.0001 |
| Undifferentiated ^b | 29 | 37 | |
| Tumor invasion | | | |
| T1a+T1b | 23 | 4 | 0.0111 |
| T2+T3+T4 | 68 | 47 | |
| Lymph node metastasis | | | |
| Positive | 5 | 11 | 0.0037 |
| Negative | 86 | 40 | |
| Liver metastasis | | | |
| Positive | 0 | 5 | 0.0024 |
| Negative | 91 | 46 | |
| Lymphatic invasion | | | |
| Positive | 50 | 42 | 0.0010 |
| Negative | 41 | 9 | |

NS: not significant. ^aDifferentiated type corresponds to well and moderately differentiated tubular and papillar tumors (intestinal type). ^bUndifferentiated type includes poorly differentiated and signet-ring cell carcinomas (diffuse type).

142 cases. TUNEL-positive nuclei were clearly seen in TP53INP1-positive (Figure 3A) and negative (Figure 3B) cancer lesions. As shown in Figure 3C, the apoptotic index in the TP53INP1-positive group ($7.48 \pm 2.66\%$) was significantly higher than that in the TP53INP1-negative group ($4.16 \pm 2.41\%$).

TP53INP1 expression and prognosis

On univariate analysis, patient survival according to

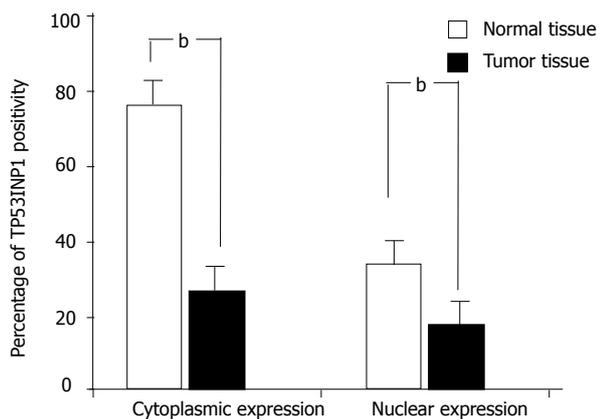


Figure 2 Comparison of TP53INP1 expression (in cell cytoplasm and nucleus) between normal gastric mucosa and gastric cancer tissues ($P < 0.0001$).

Table 3 Multivariate survival analysis using the Cox regression model

| Factor | Reference | Odds Ratio | ρ |
|---------------------|-------------------------|------------|--------|
| TP53INP1 | + vs - | 1.250 | 0.3680 |
| Age | <60 vs \geq 60 | 1.388 | 0.1077 |
| Gender | Male vs female | 1.158 | 0.5217 |
| Location | Body+cardia vs antrum | 1.253 | 0.4847 |
| Histological type | Poor vs well+moderately | 2.043 | 0.0026 |
| Tumor invasion | T2+T3+T4 vs T1 | 1.061 | 0.8485 |
| Stage | III+IV vs II+I | 1.269 | 0.2612 |
| Apoptotic index | \leq 4% vs >4% | 2.244 | 0.0008 |
| Metastasis | + vs - | 18.688 | 0.0007 |
| Lymphatic invasion | + vs - | 0.721 | 0.2124 |
| Lymph node invasion | + vs - | 3.121 | 0.0032 |

pathological stage was significantly different between TP53INP1-positive and TP53INP1-negative groups. Those patients with TP53INP1-positive expression had significantly better survival than those without TP53INP1 expression ($P = 0.0006$, Figure 4A). Survival for TP53INP1-positive patients with poorly differentiated adenocarcinoma was significantly longer than that of TP53INP1-negative patients ($P = 0.0199$, Figure 4B), whereas the survival of TP53INP1-positive patients in well or moderately differentiated adenocarcinoma was not significantly different from that of TP53INP1-negative patients ($P = 0.1110$, Figure 4C). Taken together, the results indicated that alteration of TP53INP1 expression was associated with a poor prognosis. Nevertheless, no prognostic value for TP53INP1 expression was evidenced from the multivariate analysis (Table 3). Histological type, apoptotic index, metastasis, and lymph node invasion were the most important independent prognostic factors, TP53INP1 could not be considered as an independent prognostic marker.

P53 was not expressed in non-malignant gastric tissues but expressed in gastric cancer tissues

As shown in Table 1 and Figure 1E, the p53 protein was expressed in gastric cancer regions. whereas it was absent in non-malignant portions. The staining was nuclear. Cytoplasmic staining without nuclear staining was regarded

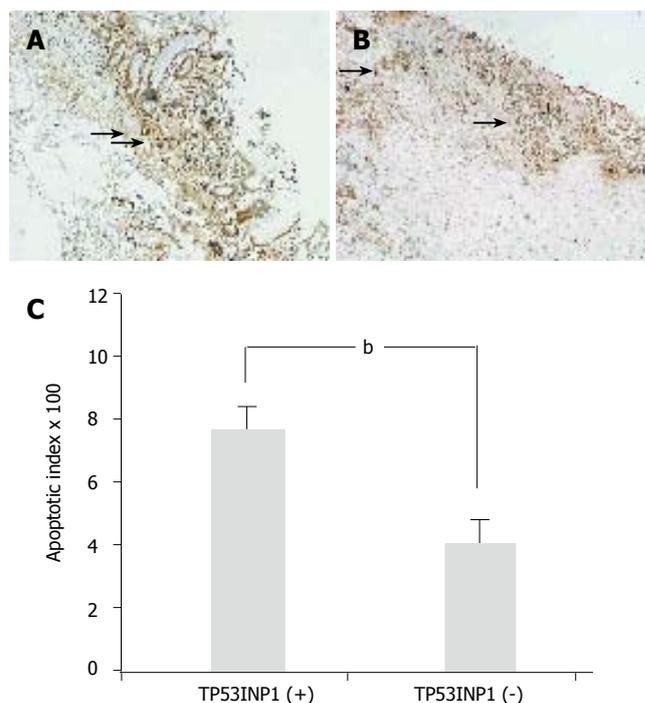


Figure 3 Apoptosis analysis in gastric cancer. **A and B:** Representative patterns of TP53INP1-positive (**A**) and negative (**B**) carcinoma in TUNEL staining. Arrows indicate TUNEL-positive nuclei (original magnification $\times 20$); **C:** Statistical analysis of apoptotic index in TP53INP1-positive and -negative cancer tissues ($P < 0.0001$, positive vs negative).

as negative. The p53 positive rate in gastric cancer was significantly higher (47.9%) than that in non-malignant tissues ($P < 0.05$).

DISCUSSION

The development and progression of gastric cancer involve many types of genes that need to be activated or inactivated in order to promote malignancy. Gastric cancer is a heterogeneous pathology, classified into two general subtypes: intestinal (differentiated) and diffuse (undifferentiated)^[17]. The intestinal type gastric carcinoma presents tumor suppressor gene alterations similar to colorectal tumors and distinct from diffuse type gastric cancer^[18]. An accumulation of multiple genetic and epigenetic alterations of oncogenes, tumor suppressor genes, DNA repair genes, cell cycle regulators, cell adhesion molecules, and growth factor/receptor systems are involved during the multi-step conversion from normal epithelial cells to clinical gastric cancer^[10-14]. TP53 gene alterations have been observed in both histological subtypes^[19]. TP53INP1 is a tumor suppressor gene, located on the chromosome band 8q22^[20]. Its expression is dependent on the activation of wide-type p53^[3].

In this study, we have showed that TP53INP1 protein expression was significantly reduced in gastric cancer cells compared with non-cancerous adjacent tissues. We also reported that reduced TP53INP1 expression was associated with the diffuse cancer phenotype. Tomasini *et al*^[2] have shown that TP53INP1 and HIPK2 are partners in regulating p53 activity. It is increasingly evident that methylation of CpG islands in the promoter of specific

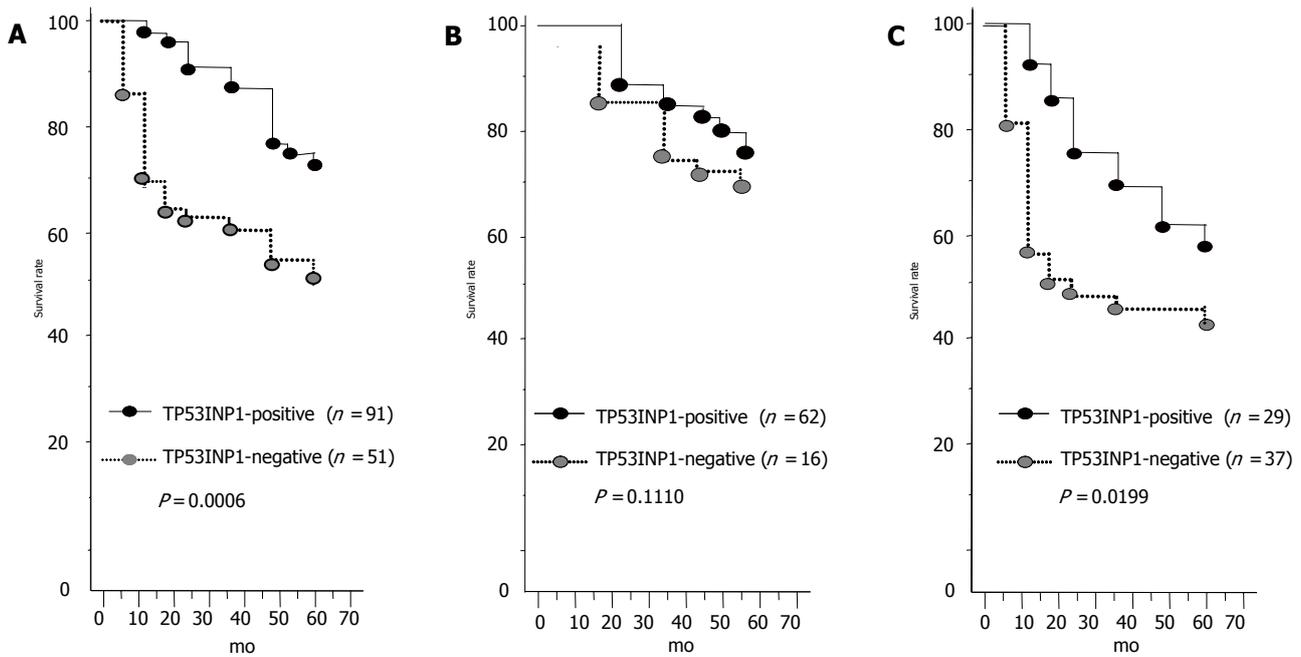


Figure 4 TP53INP1 expression and patient survival by Kaplan-Meier analysis. **A:** Survival curves for TP53INP1-positive and negative gastric cancers. The 60-mo survival rates were 74.7% and 54.9%, respectively. The difference between the values was highly significant (P=0.0006). **B:** In well or moderately differentiated adenocarcinoma (intestinal-type), the survival of TP53INP1-positive patients was not significantly different from that of TP53INP1-negative group (P=0.1110). **C:** In poorly differentiated adenocarcinoma (diffuse-type), the 60-mo patient survival rates were 62.1% and 45.9% for TP53INP1-positive and -negative gastric cancers, respectively. This difference was statistically significant (P=0.0199).

tumor suppressor genes, such as *p16*, is associated with their silencing in human gastric cancer^[21]. The 5'-upstream region of TP53INP1 contains a CpG island. The sequences from nucleotide -792, the region on exon 1 and part of the first intron, to nucleotide +839 have the highest content of CpG dinucleotides. However, there was no mutation of TP53INP1 gene in pancreatic carcinoma (unpublished data). In addition, the reduced TP53INP1 expression in gastric cancer especially in the diffuse type, may relate to the wide-type p53 inactivation in gastric cancer.

We showed that p53 was expressed in 68 cases of gastric cancer, whereas it was not present in normal gastric epithelial cells. We observed that in non-gastric cancer regions the expression of TP53INP1 was opposite to that of p53. The expression of TP53INP1 was dependent on the wild type p53 since the wild-type p53 protein is biologically unstable and has a shorter half-life than mutant p53 protein^[24]. This characteristic of wide-type p53 protein does not allow it to be detected by immunohistochemical methods, but mutant p53 can be detected by immunostaining. Our data was similar to Carvalho *et al*^[22] who showed that there was no difference of the p53 expression between the intestinal type and the diffuse type in gastric cancer. Whereas Lin *et al*^[23] showed the expression of p53 in the intestinal type was more frequent than that in the diffuse type. The precise mechanisms of TP53INP1 suppression in gastric cancer need further research.

The mechanism of the suppression of these genes in poorly differentiated gastric carcinoma is not clear. Many studies suggested that most tumor suppressor genes play a role in mediating cell cycle arrest in the

G₁ phase following DNA damage and also function in the removal of damaged cells by initiating apoptosis in certain physiological situations^[10]. TP53INP1 and HIPK2 are partners in regulating p53 activity^[2]. Overexpression of TP53INP1 induces G₁ cell cycle arrest. TP53INP1 expression was significantly decreased in advanced gastric cancers. These results suggest that decrease of TP53INP1 expression might play an important role in the progression of gastric cancer. Assessment of TP53INP1 expression level may serve as a novel biomarker for predicting the malignant grade of cancers, like another marker for poor prognosis genes^[25,26].

Lymphatic involvement is thought to be important as an initial step of lymph node metastasis^[27]. Our study showed that TP53INP1 expression was significantly reduced in lymphatic invasion-positive groups. TP53INP1 expression decreased in node-positive. Taken together, these results suggested that loss of TP53INP1 expression is associated with lymph node metastasis of gastric cancer.

Deregulation of genes involved in cell cycle and cell signaling pathways has been described and classified as early events for cyclin D1 and p16 genes or late events for p53, DPC4 and BRCA2 genes in the progression model studies^[28]. TP53INP1 is a pro-apoptotic gene strongly activated during cell stress. Overexpression of TP53INP1 is related to G₁ cell cycle arrest and induces p53-mediated cell death^[3]. In the present study, we showed that the apoptotic index in TP53INP1-positive lesions was higher than that in TP53INP1-negative lesions in gastric cancer tissues as detected by TUNEL assay, indicating that TP53INP1 is related to apoptosis and tumor aggressiveness in gastric carcinoma.

TP53INP is an acute gene that is induced by various

stresses such as UV, heat shock, etc.^[1]. Up to now, we have known little function of it. We for the first time revealed the impact of the TP53INP1 on the survival in gastric cancer. We showed that the survival rate of the TP53INP1-positive cases was longer than that of the TP53INP1-negative cases, especially in the diffuse type gastric cancer.

In conclusion, the present study has showed that the reduction of TP53INP1 expression might play roles in gastric carcinogenesis and tumor aggressiveness. Analysis of the TP53INP1 may be useful to evaluate the malignant grade of gastric cancer.

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Cyclosporine *versus* tacrolimus in patients with HCV infection after liver transplantation: Effects on virus replication and recurrent hepatitis

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CONCLUSION: Cyclosporine or tacrolimus as a primary immunosuppressive agent does not influence the induction or severity of recurrent hepatitis in HCV-infected patients after liver transplantation.

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Key words: Cyclosporine; Tacrolimus; Liver transplantation; Recurrent hepatitis; HCV-RNA

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Abstract

AIM: To determine the effects of the calcineurin inhibitors, cyclosporine and tacrolimus, on hepatitis C virus (HCV) replication and activity of recurrent hepatitis C in patients post liver transplantation.

METHODS: The data of a cohort of 107 patients who received liver transplantation for HCV-associated liver cirrhosis between 1999 and 2003 in our center were retrospectively analyzed. The level of serum HCV-RNA and the activity of recurrent hepatitis were compared between 47 patients who received either cyclosporine or tacrolimus as the primary immunosuppressive agent and an otherwise similar immunosuppressive regimen which did not lead to biliary complications within the first 12 mo after transplantation.

RESULTS: HCV-RNA increased within 3 mo after transplantation but the differences between the cyclosporine group and the tacrolimus group were insignificant ($P=0.49$ at 12 mo). In addition, recurrent hepatitis as determined by serum transaminases and histological grading of portal inflammation and fibrosis showed no significant difference after 12 mo ($P=0.34$).

INTRODUCTION

Hepatitis C virus (HCV) is a leading cause of chronic hepatitis with about 170-190 million people infected worldwide^[1,2]. The treatment of choice for patients with end-stage liver disease associated with HCV infection is liver transplantation (LTx) and in fact, HCV is the main indication for liver transplantation among adults in Europe and USA, accounting for approximately 50% of cases^[3]. Since HCV persists not only in hepatocytes but also in leukocytes, lymph nodes and most likely additional tissues^[4-6], the virus is redistributed into the donor liver after LTx almost immediately in virtually all patients^[7,8]. However, the severity and clinical course of the resulting reinfection hepatitis vary widely, ranging from deleterious fibrosing cholestatic hepatitis with failure of the allograft within 1 year to a rather mild hepatitis with progression to cirrhosis within 5 years in 20-30% of cases^[9,10].

A main factor determining the severity of recurrent hepatitis C after transplantation may be immunosuppression^[11]. Of particular concern is the fact that the prognosis of recurrent HCV infection seems to deteriorate in recent years, which has been attributed to the use of donor organs of less optimal quality as well as changes in the immunosuppressive therapy such as steroid-free regimens

or the combination of particular drugs^[12,7]. Considering that retransplantation remains the only treatment for patients with graft failure due to HCV recurrence, optimization of the immunosuppressive regimens might be a key aspect to improve the prognosis of chronic hepatitis C after transplantation.

The two most frequently used basic immunosuppressive drugs are cyclosporine A (CsA) and tacrolimus (Tac). Although CsA and Tac are structurally different, they share a similar mode of immunosuppressive action by inhibition of the transcription factor NFAT and consecutive reduction of inflammatory gene expression in activated T cells^[13-15]. The main mechanism by which immunosuppressive therapy with calcineurin inhibitors accelerates the clinical course of post-transplantation hepatitis C is their influence on the replication of the virus. Immunosuppressive therapy after LTx of patients with chronic HCV infection is associated with significantly increased serum viral load compared to the values of the same patients before transplantation^[7,11], suggesting decreased systemic immune responses against the virus. Locally in the liver, the uncontrolled replication of the virus may cause an enhanced immune response, resulting in accelerated hepatocellular damage and fibrosis.

Whether a particular calcineurin inhibitor has an advantage in immunosuppressive therapy of HCV-infected patients post LTx is still highly controversial^[16-19]. To further clarify this issue, the aim of this study was to determine the effects of CsA and Tac on serum HCV-RNA level as well as on the inflammatory activity of recurrent hepatitis in patients with chronic HCV infection after LTx.

MATERIALS AND METHODS

Patients

The data of 107 consecutive patients within the first year after liver transplantation due to hepatitis C-associated liver cirrhosis were retrospectively reviewed. Patients underwent LTx between June 1998 and December 2003 at our center either as cadaveric-(orthotopic) or as living donor-related liver transplantation. In order to compensate for missing randomization in this retrospective study, only 47 patients who received either cyclosporine or tacrolimus as the primary immunosuppressive agent and an otherwise similar immunosuppressive regimen which did not lead to acute or chronic rejection and biliary complications within the first 12 mo after transplantation, forming a relatively homogenous subgroup, were included in this study. A further exclusion criterion was early death after transplantation. During the first year all patients underwent routine liver biopsy 3-5 mo after LTx, independent from serum liver enzyme activities. Every rise of transaminases or cholestasis parameters was followed by additional liver biopsies to differentiate between acute or chronic rejection, cholestasis and hepatitis C reinfection. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Local Ethics Committee.

The basic immunosuppressive agent used in all the patients was either CsA or Tac. The dose of the drugs was

controlled in light of the serum level, which was adjusted between 140 and 180 ng/mL for CsA, and 10 and 15 ng/mL for Tac within the first 3 mo. After 3 mo, the serum level of CsA and Tac was reduced to 100-120 ng/mL and 7-10 ng/mL, respectively. During the first 3 mo. after LTx, CsA, and Tac were routinely combined with corticosteroids (prednisolone) at an initial dose of 1 mg/kg body weight and reduced to 5 mg daily within 4 weeks. After 3 mo steroids were discontinued. In addition, all the patients included in this study received 1000-2000 mg mycophenolate mofetil (MMF), per day for the first 12 mo post LTx.

Determination of HCV infection

HCV infection status was evaluated by the examination of the patients' sera for the presence of anti-HCV antibodies and HCV-RNA. Anti-HCV antibodies were detected by a commercially available enzyme immunoassay (Monalisa anti-HCV plus, Biorad, Munich) according to the manufacturer's instructions. Qualitative detection of serum HCV-RNA was performed by a RT-PCR-based (Cobas AmpliCor HCV 2.0 assay, Roche Diagnostics, Mannheim, Germany) assay or a transcription-mediated amplification (TMA)-based assay (VERSANT HCV-RNA qualitative, Bayer Diagnostics, Fernwald, Germany). HCV-RNA concentrations were determined by commercially available second and third generation branched DNA (bDNA) assays (VERSANT HCV-RNA 2.0 and 3.0, Bayer Diagnostics, Fernwald, Germany). To obtain HCV-RNA titers in IU/mL, the bDNA 2.0 assay was calibrated against the WHO HCV Standard 96/790^[20], as described in full details elsewhere^[21,22]. Values below the lower limit of sensitivity of 615 IU/mL were set to 615 IU/mL divided by 2.

Determination of inflammatory activity

Inflammatory activity in the graft due to hepatitis induced by HCV reinfection was in all patients under surveillance determined by routine measurement of transaminases (ALT or GPT and AST or GOT) as well as alkaline phosphatase and gamma-glutamyl-transferase and bilirubin (every 7-30 d, interval depending on the time after LTx and level of elevation). Every unexplained rise was immediately followed by B-mode and duplex-/Doppler ultrasonographic analysis of liver parenchyma and the supplying vessels. If thrombosis of the hepatic artery and congestion of the biliary system were excluded, liver biopsy was performed. In analogy to patients pre LTx, recurrent hepatitis C severity was routinely determined by the modified Knodell hepatic activity index (HAI) with subscores for piecemeal necrosis (0-4), confluent necrosis (0-6), lytic necrosis/apoptosis/focal inflammation (0-4) and portal inflammation (0-4), as well as fibrosis staging (0-6)^[23].

Statistical analysis

Virological, biochemical, and histological data were collected in a computer database (Microsoft Excel). Statistical analyses were performed utilizing SAS System software, release 8.2 (SAS Institute Inc., Cary, NC, USA). Qualitative characteristics were analyzed by χ^2 test or

Table 1 Baseline characteristics of 47 HCV-infected patients after LTx, orthotopic liver transplantation (OLT), and living donor liver transplantation (LDLT)

| | CsA | Tac | P-value |
|--------------------|-------------------------------------|-------------------------------------|---------|
| Number of patients | 35 | 12 | |
| Age | 28-64 | 27-61 | 0.20 |
| Gender | f=9, m=26 | f=3, m=9 | 0.96 |
| OLT | 31 | 10 | |
| LDLT | 4 | 2 | 0.64 |
| HCV genotype | gt1=32/35, gt2=1/35, gt3=2/35 | gt1=10/12, gt2=0/12, gt3=2/12 | 0.46 |

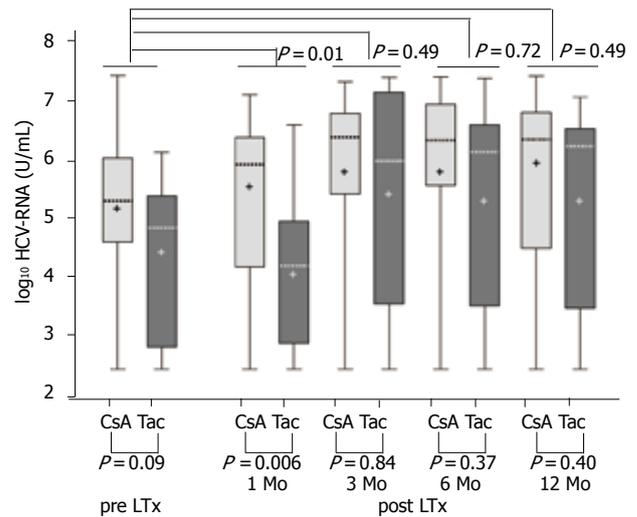
Fisher's exact test as appropriate. Two-sided *P*-values and changes in HCV-RNA concentrations and ALT activities were evaluated by Mann-Whitney *U*-test. Correlation coefficients were reported as rank (Spearman) correlations. $P < 0.05$ was considered statistically significant. In this explorative analysis, no adjustment for multiple testing was performed.

RESULTS

Effect of CsA and Tac on HCV serum RNA levels in HCV-infected patients post LTx

Of the 47 patients included in this study, 35 received CsA and 12 received Tac as the primary immunosuppressive agent. The baseline characteristics of the two patient groups showed no statistically significant differences (Table 1). Patients underwent liver transplantation at least 12 mo before the study. In both groups of patients, serum HCV-RNA level was quantitatively assessed at 1, 3, 6, and 12 mo after liver transplantation and compared to the HCV-RNA level prior to the operation. Figure 1 depicts these data on logarithmic scale (\log_{10}) as a box-whisker's plot of HCV-RNA in relation to the primary immunosuppressive drug (CsA or Tac) and the time after LTx. *P*-values (Mann-Whitney *U*-test) at the top of the Figure express the group differences of changes in HCV serum RNA level (virus load), after 1, 3, 6, and 12 mo and the last measurement pre transplantation as indicated. *P*-values at the bottom of the Figure are the group differences between HCV-RNA concentrations at the given time of measurement. Boxes indicate the lower and upper quartiles and median, '+' indicates mean.

Serum HCV-RNA concentrations showed a substantial rise (between 10- and 1000-fold) after LTx. The elevation of HCV-RNA levels developed between 1 and 3 mo post LTx, at which point the elevation was the most pronounced and continued then throughout the entire observation period of 12 mo. The patients who received tacrolimus for immunosuppression had a lower mean HCV-RNA level pre LTx than those who received CsA, but the difference was not statistically significant ($P = 0.09$). One month post LTx this group difference in the HCV-RNA levels was still present and even slightly accentuated, reaching statistical significance at this point ($P = 0.006$). However, 3 mo post LTx the difference was significantly diminished neither at this time ($P = 0.84$) nor at 6 ($P = 0.37$) or 12 mo ($P = 0.40$) post LTx. The group difference reached statistical significance.

**Figure 1** Box-whisker's plot of \log_{10} -transformed HCV-RNA concentrations in dependence on group (immunosuppression) and time.

A statistical significance between the levels of HCV-RNA in CsA and Tac groups was evaluated by comparison of the group difference at each given time point after LTx with the group difference before LTx. This analysis revealed no relevant dissimilarity between serum HCV-RNA concentrations in patients who received CsA or Tac for immunosuppression. A slightly higher rise in the tacrolimus group particularly 3 mo post LTx was statistically insignificant ($P = 0.49$). The differences at 6 and 12 mo between both the groups were also not significant ($P = 0.72$, $P = 0.49$). Table 2 lists the net group differences of the changes on a \log_{10} -scale in serum HCV-RNA between the pre-LTx and post LTx values.

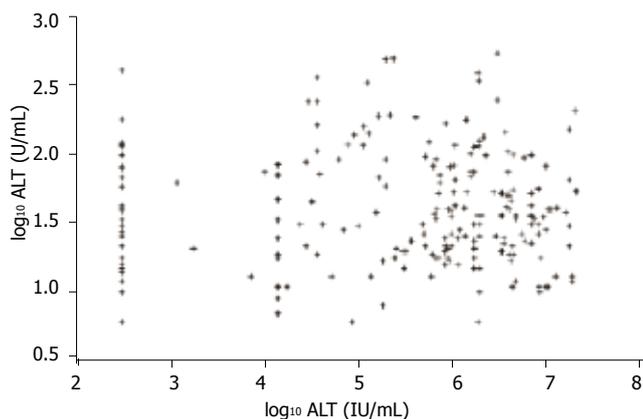
A power analysis revealed that with the given patient number, the group differences of the changes in serum HCV-RNA concentrations should be on the order 10-fold higher in order to be detectable with a power of 80%. In fact, the strongest deviation from the null hypothesis of no group difference of changes was not a significantly ($P = 0.11$) stronger increase by a factor of 2.5 from pre LTx to the 1 mo measurement in the CsA as compared to the tacrolimus group, opposing a possible superiority of CsA.

Activity of recurrent hepatitis C in dependence of immunosuppression

The value of HCV serum RNA level in predicting the inflammatory activity and progression of fibrosis was analyzed by a scatter plot of serum ALT, mirroring the activity of recurrent hepatitis, in dependence of serum HCV-RNA levels (Figure 2). The correlation coefficient was almost equal to zero ($r = 0.01$, $P = 0.92$), suggesting no significant association between levels of HCV-RNA and intrahepatic inflammatory activity. Therefore HCV-RNA levels post transplantation may not be a valid predictor of allograft inflammation. Differences in the progression of recurrent hepatitis were examined by serum transaminase level and histology in the two patient groups. Figure 3 shows the ALT levels (logarithmically scaled) as a box-whisker's plot of enzyme activity in correlation to

Table 2 Net group difference (CsA vs FK 506) of changes in virus load (\log_{10} -scale) between pre-LTx measurement and indicated time points post LTx

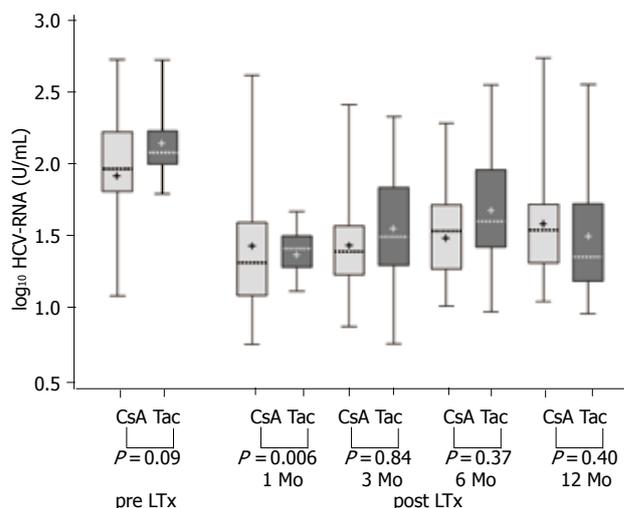
| Mo | Mean | 95%CI | P-value ¹ |
|----|-------|---------------|----------------------|
| 1 | 0.40 | (-0.28; 1.08) | 0.11 |
| 3 | -0.50 | (-1.46; 0.47) | 0.49 |
| 6 | -0.43 | (-1.50; 0.64) | 0.72 |
| 12 | -0.56 | (-1.64; 0.52) | 0.49 |

¹Mann-Whitney U test.**Figure 2** Correlation of HCV-RNA level in serum with ALT activity. Scatter plot with HCV-RNA concentrations on the y-axis and ALT activity on the x-axis, both on \log_{10} -scale.

immunosuppression (CsA group or FK 506 group) and the time after LTx. P-values (Mann-Whitney U-test) at the bottom of the Figure are for group differences between ALT activity at the time of measurement. Boxes indicate the lower and upper quartiles and median, '+' indicates mean.

In comparison to the level before LTx, the ALT level fell significantly immediately after the operation, with the lowest values 1 mo post LTx. After 3 mo especially after 6 mo ALT levels rose again and stayed elevated over the entire observation period of 12 mo, indicating the development of recurrent chronic hepatitis C. The ALT activity after 3 and 6 mo was increased in the CsA group, but the difference was not statistically significant (3 mo: $P=0.36$, 6 mo: $P=0.65$). Analysis of AST activity revealed similar kinetics post LTx with lower absolute values and also no statistically significant group differences (data not shown).

In conjunction with these analyses, examination of the proportion of patients with normal activities of transaminases post LTx revealed that 70% of patients in the CsA group and 83% in the Tac group had normal ALT activities 1 mo post LTx (Figure 4A). Recurrent hepatitis led to a significant decrease of the proportion of patients with normal ALT values after 3 and 6 mo (48% in the CsA group and 42% in the Tac group). While the proportion of patients with normal ALT at these time points was slightly higher in the CsA group than in the Tac group which was reversed after 12 mo, only 49% of patients in

**Figure 3** Box-whisker's plot of \log_{10} -transformed ALT activities in dependence on group (immunosuppression) and time.

the CsA group but 58% of patients in the Tac group had normal ALT values. However, the group differences were statistically significant neither at 6 mo ($P=0.69$) nor at 12 mo ($P=0.56$). The proportion of patients with normal AST changed similarly (Figure 4B), indicating no advantage for CsA in suppressing inflammatory activity.

To further evaluate the potential differences in the activation and maintenance of recurrent hepatitis C between the two groups, we compared necroinflammatory activity and grade of fibrosis according to HAI scores in the liver histology taken 3-5 mo post LTx. The majority of patients in both groups had a grade II inflammation (74% of patients in the CsA group and 83% of patients in the Tac group), indicating that recurrent hepatitis occurred in a significant proportion of patients in the absence of elevated serum transaminases (Figure 5A). The total group differences including all inflammation grades between the CsA and Tac groups were statistically negligible ($P=0.85$). Examination of fibrosis revealed a larger proportion of patients in the CsA group than in the Tac group scored with grade 0 (26% vs 8.3%, Figure 5B). However, at grade I this relation was reversed (2.9% vs 25%). In addition, more patients in the CsA group were scored with grade II fibrosis, while patients in the Tac group were more frequently scored with grade III fibrosis. The number of patients presenting with grade IV fibrosis was very small in both groups. Due to the changes in grades 0 and I, these overall group differences were statistically significant ($P=0.03$). However, if grades 0 and I were analyzed as one group, no statistical differences were found ($P=0.34$).

DISCUSSION

The marked increase in serum HCV-RNA levels after LTx is closely associated with the immunosuppressive therapy^[7,11,24]. Our study also confirmed that serum HCV-RNA levels increased in the early (3 mo) post-transplant period, when immunosuppression was the highest. Although there is evidence that immunosuppression accelerates the progression of chronic HCV infection to cirrhosis, no clear

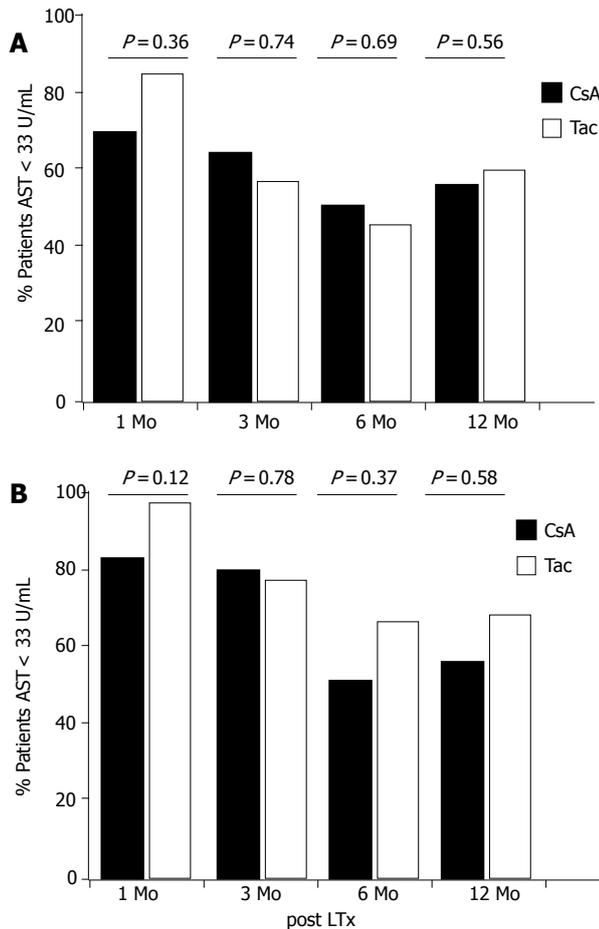


Figure 4 Proportion of HCV-infected patients under CsA or Tac medication with normal transaminase activities at different time points post LTx. **A:** Percentage of patients with normal ALT; **B:** percentage of patients with normal and AST. *P*-values were calculated by χ^2 test.

association has been found between the type or dose of immunosuppressive regimen and the outcome of post-transplant HCV recurrence. CsA and Tac are the most common immunosuppressive drugs used after LTx, but their exact impact on the incidence, severity, time of onset and outcome of HCV recurrence is unclear^[10]. In this context, Watashi *et al.*^[17] showed that CsA but not Tac can inhibit HCV replication *in vitro*, which raises the question whether HCV-infected patients treated with CsA have an advantage with regard to the progression of recurrent hepatitis and therefore graft survival as compared to those treated with Tac.

Our results did not confirm the function of CsA in inhibiting HCV replication as compared to Tac. Neither the differences at any time of examination nor the differences in serum HCV-RNA levels after LTx were in favor of CsA. In addition, there were no significant differences in inflammation of the allograft between the CsA and Tac groups, as determined by serum transaminase activities and histological grading of intrahepatic necroinflammatory activity. The slight differences in the grade of fibrosis, suggesting a lower grade of fibrosis in the CsA group were not statistically significant. While a study showed that HCV-infected patients treated with Tac have an improved survival in comparison to those treated with CsA^[16], our results are in accordance with a recently published study, which also

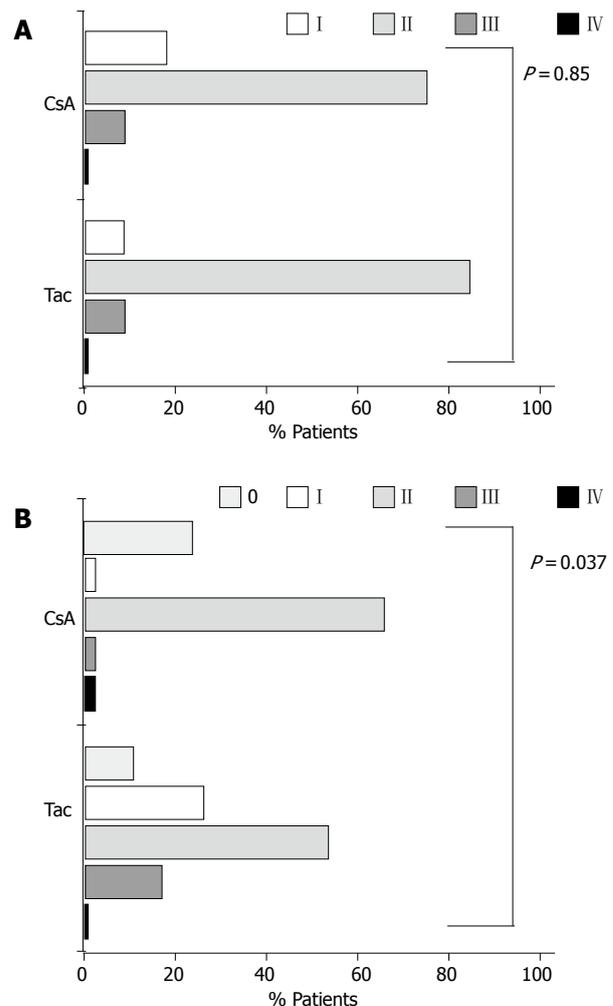


Figure 5 Intrahepatic inflammatory activity and fibrosis post LTx depending on immunosuppression. **A:** Necroinflammatory activity histologically scored by the subscores for portal inflammation of the Knodell's hepatic activity index (HAI); **B:** grade of fibrosis in the graft. Fibrosis was scored according to the appropriate subscores of the HAI.

found no advantage for either immunosuppressive agent^[19]. However, Watashi *et al.*^[17] showed that CsA can inhibit HCV replication in hepatocytes. In the last several years, a number of cell culture propagation systems based on the infection of primary human^[25,26] or chimpanzee^[27] hepatocytes or hepatic cell lines^[28,29] have been reported. There is a general agreement that all these systems have a poor validity and reproducibility. Viral proteins and particles have never been convincingly detected and viral RNA can be quantified only by the sensitive PCR methodology^[30,31]. In HCV research, these limitations of the infection-based cell culture systems have led to the development of a selectable HCV replicon system^[32]. In this system, a subgenomic part of HCV-RNA can replicate in a human hepatoma cell line (HuH-7) to unphysiologically high levels, suggesting that this system is highly artificial and may not adequately mirror the situation *in vivo*. An additional important aspect regarding both cell culture systems is the absence of the immune system. Even if CsA has a HCV suppressing effect *in vivo*, this effect is by far outweighed by the immunosuppressive effects of the drug, resulting in a decreased control of HCV replication by the immune system.

In conclusion, our study does not justify a recommendation for CsA as a primary immunosuppressive agent for the treatment of HCV-infected patients post LTx. Further prospective studies should be conducted to clarify this issue. To vigorously improve the graft survival and prognosis of HCV-infected patients post LTx, development of new therapeutic agents directly modifying HCV-replication and/or inflammatory activity of recurrent hepatitis C in combination with alternative immunosuppressive regimens remains a high priority goal.

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STI571 (Glivec) suppresses the expression of vascular endothelial growth factor in the gastrointestinal stromal tumor cell line, GIST-T1

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Abstract

AIM: To estimate whether STI571 inhibits the expression of vascular endothelial growth factor (VEGF) in the gastrointestinal stromal tumor (GIST) cells.

METHODS: We used GIST cell line, GIST-T1. It has a heterogenic 57-bp deletion in exon 11 to produce a mutated c-KIT, which results in constitutive activation of c-KIT. Cells were treated with/without STI571 or stem cell factor (SCF). Transcription and expression of VEGF were determined by RT-PCR and flow cytometry or Western blotting, respectively. Activated c-KIT was estimated by immunoprecipitation analysis. Cell viability was determined by MTT assay.

RESULTS: Activation of c-KIT was inhibited by STI571 treatment. VEGF was suppressed at both the transcriptional and translational levels in a temporal and dose-dependent manner by STI571. SCF upregulated the expression of VEGF and it was inhibited by STI571. STI571 also reduced the cell viability of the GIST-T1 cells, as determined by MTT assay.

CONCLUSION: Activation of c-KIT in the GIST-T1 regulated the expression of VEGF and it was inhibited by STI571. STI571 has antitumor effects on the GIST cells with respect to not only the inhibition of cell growth, but also the suppression of VEGF expression.

INTRODUCTION

Gastrointestinal stromal tumors (GIST) are characterized by the expression of both c-KIT and CD34 on the cell surface. Mutations in *c-kit* have recently been implicated with oncogenic activation associated with GISTs^[1,2]. c-KIT, a type III receptor tyrosine kinase, is activated upon binding of the ligand, stem cell factor (SCF) to initiate a signaling pathway that is critical for the growth and development of mast cells, melanocytes, hematopoietic stem cells, and the interstitial cells of Cajal^[3,4]. Gain-of-function mutations in *c-kit* are associated with a number of cancers in human beings^[1,5-9]. The majority of GISTs show constitutive c-KIT phosphorylation due to a gain-of-function mutation in exon 11 (cytoplasmic juxtamembrane domain), with other mutations known to occur in exon 9 (extracellular membrane domain), exon 13 (first part of the split tyrosine kinase domain), and exon 17 (phosphotransferase domain)^[8].

STI571 is a specific tyrosine kinase inhibitor that acts on Bcr-Abl, platelet-derived growth factor receptor (PDGFR), and c-KIT. STI571 has been used successfully in patients with unresectable or metastatic GISTs that show constitutive activation of c-KIT^[9].

Vascular endothelial growth factor (VEGF) is a highly specific mitogen for vascular endothelial cells that is induced by hypoxia, oncogene activation, and a variety of cytokines. VEGF is important in angiogenesis and neovascularization of solid tumor growth^[10]. Expression of VEGF in GISTs was reported by Takahashi *et al*^[11], who suggested a correlation between this expression

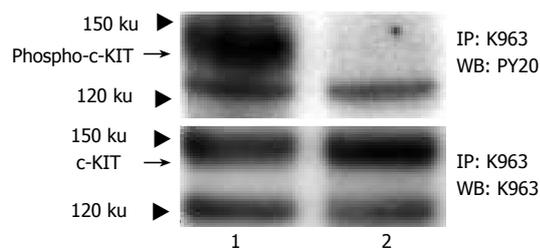


Figure 1 4×10^6 of GIST-T1 cells were treated with STI571 (1 $\mu\text{g}/\text{mL}$) for 1 h. Treated cells were lysed in RIPA buffer and immunoprecipitation with anti-c-KIT antibody as described in "Materials and methods". Western blotting was performed using anti-phosphotyrosine antibody (upper panel) or anti-c-KIT antibody (lower panel). Lane 1, non-treated; lane 2, STI571 1 $\mu\text{g}/\text{mL}$. WB, Western blot; IP, immunoprecipitation.

and poor prognosis. It is thought that the assessment of VEGF expression in GISTs is important clinically.

In the present study, we have analyzed the expression of VEGF in the GIST cell line, GIST-T1, with or without STI571 treatment. To our knowledge, there are only two GIST cell lines, including GIST-T1. The GIST-T1 line was established from a patient with metastatic GIST^[12], which showed the *c-kit* mutation in exon 11. In addition, we have analyzed the effect of STI571 on cell growth of the GIST-T1 cells.

MATERIALS AND METHODS

Materials

STI571, also known as Glivec capsule (Novartis, Basel, Switzerland), was dissolved in water (5 $\mu\text{g}/\mu\text{L}$) and stored at -20°C . Antibodies were used to detect the c-KIT (K963, rabbit polyclonal IgG, Immuno-Biological Laboratories, Japan), phosphotyrosine (PY20, mouse monoclonal IgG, Zymed, USA), HIF-1 alpha (H-206, rabbit polyclonal IgG, Santa Cruz Biotechnology, USA), and VEGF (A-20, rabbit polyclonal IgG, Santa Cruz Biotechnology, USA).

Cells and cell culture

The human GIST cell line, GIST-T1 has been characterized in detail by Taguchi *et al*^[12]. GIST-T1 cells and DLD-1 cells (colon adenocarcinoma cell line) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin, streptomycin, and 80 mL/L fetal bovine serum maintained in a 50 mL/L CO_2 atmosphere at 37°C in a humidified incubator.

Western blot analysis

Cells were washed thrice with ice-cold phosphate buffered saline (PBS) and then lysed in RIPA buffer containing 20 mmol/L sodium pyrophosphate, 20 mmol/L NaF, 1 mmol/L orthovanadate, 2 mmol/L pyrophosphate, 1 mmol/L PMSF, 10 $\mu\text{g}/\text{mL}$ aprotinin, and 10 $\mu\text{g}/\text{mL}$ leupeptin. Cell lysates containing comparable amounts of proteins, estimated by a Bradford assay (BioRad, Munchen, Germany), were analyzed by Western blotting using antibodies as listed above.

Immunoprecipitation analysis

A measure of 4×10^6 cells was seeded on 10-cm dishes and

incubated for 24 h, prior to the treatment with STI571. Cells were treated with or without STI571 (1 $\mu\text{g}/\text{mL}$) for 1 h. Treated cells were collected and washed with ice-cold PBS thrice and lysed in RIPA buffer as described above. Cells lysates in RIPA buffer were subjected to immunoprecipitation with c-KIT antibody. The immunoprecipitates were reacted with protein A-agarose and washed with Tris buffered saline. They were then finally resuspended in $3 \times \text{SDS}$ sample buffer containing $30 \times \text{DTT}$ and boiled at 95°C for 5 min. Samples were separated by 75 g/L SDS-PAGE and transferred to a membrane for immunoblot analysis.

Isolation of RNA and detection of VEGF or HIF-1 alpha mRNA

A measure of 1×10^6 cells was seeded on 6-cm dishes and incubated for 24 h prior to the treatment with or without the reagents. Prior to the treatment with SCF, medium was changed to the serum-free medium. Treated cells were washed twice in ice-cold PBS and the total cellular RNA was isolated by using TRIzol reagent (Gibco-BRL) according to the manufacturer's protocol. Specific mRNA was assayed using the reverse transcription polymerase chain reaction (RT-PCR). VEGF PCR condition^[13]: denaturation at 94°C for 1 min, annealing at 60°C for 1 min and elongation at 72°C for 1 min, for 35 cycles; HIF-1 alpha^[14]: denaturation at 94°C for 10 min-1 cycle, followed by 35 cycles of 30 s at 94°C , 30 s at 55°C and 1 min at 72°C ; beta-actin: denaturing, 94°C for 30 s, annealing at 50°C for 40 s and elongation, 72°C for 1 min, for 30 cycles; and final elongation at 72°C for 10 min-1 cycle. PCR products were subjected to electrophoresis on 20 g/L agarose gel. The primer sequences were VEGF sense: 5' -CGAAGTGGTGAAGTTCATGGATG-3'; VEGF antisense: 5' -TTCTGTATCAGTCTTTCCTGGTGA-3'; HIF-1 alpha sense: 5' -CTCAAATCGGACAGCCTCA-3'; HIF-1 alpha antisense: 5' -CCCTGCAGTAGGTTTCTGCT-3'; beta-actin sense: 5'-ATTGGCAATGAGCGGTTCCGC-3'; beta-actin antisense: 5'-CTCCTGCTTGCTGATCCACATC-3'.

Flow cytometry

A measure of 2×10^6 cells was seeded on 6-cm dishes and incubated for 24 h, prior to the treatment with STI571 for 4 h. Following this, a Goldi plug (BD Bioscience, San Diego, CA, USA) was added to each dish for 2-h incubation. Cells were then washed with ice-cold PBS twice before the treatment with cytoflex (BD Bioscience) and cytoperm (BD Bioscience) according to the manufacturer's protocols. Anti-VEGF, anti-HIF-1 alpha, and FITC-labeled anti-rabbit antibodies were used. Normal rabbit IgG antibody and FITC-labeled anti-rabbit antibody were used for control staining.

MTT assay

A measure of 1×10^4 cells/100 μL was seeded in each well of a 96-well plate. After 24 h with or without STI571 treatment, 100 μL of a 2.5 mg/mL solution in PBS of the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma-Aldrich) tetrazolium substrate was added and incubated for 4 h at 37°C . The resulting violet

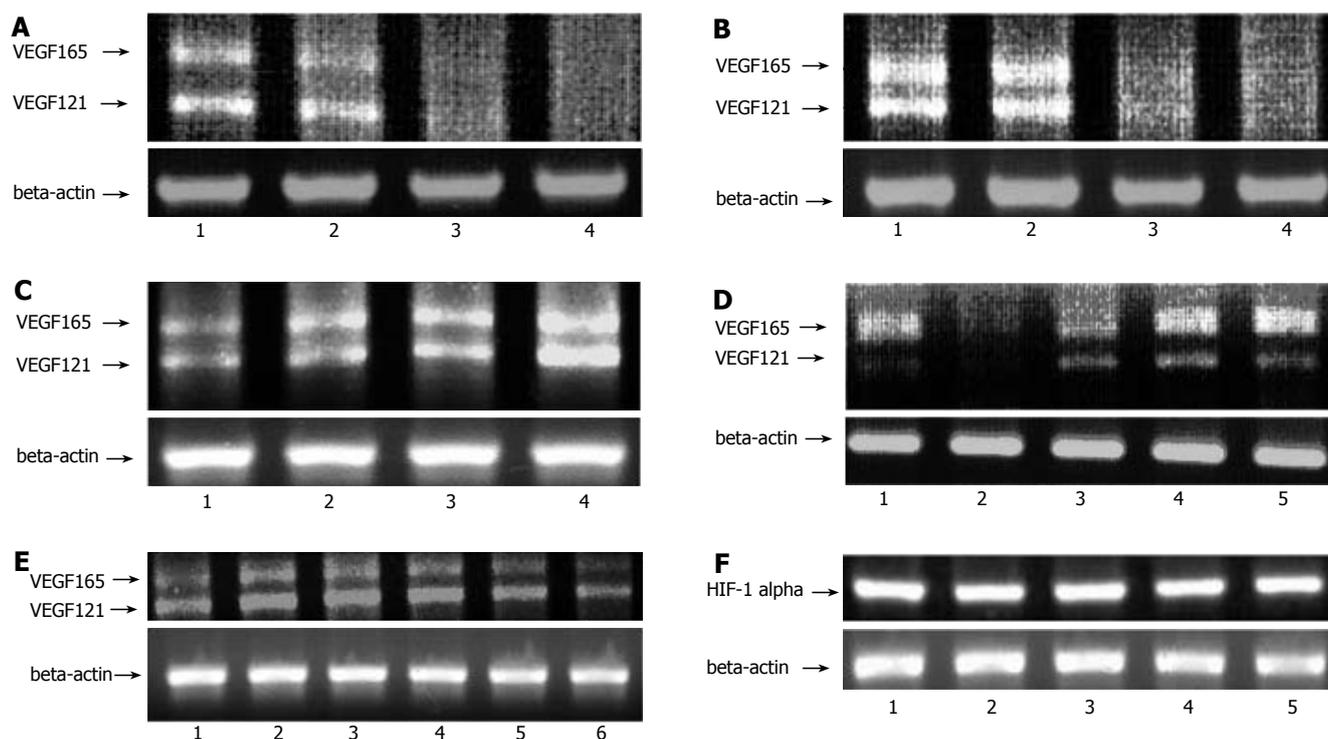


Figure 2 RT-PCR, 1×10^6 GIST-T1 cells were treated with STI571 (0.01, 0.1, and 1 µg/mL) for 6 h (A) or 1 µg/mL of STI571 for 60, 120, and 360 min (B). **A:** Lane 1, non-treated; lane 2, 0.01 µg/mL; lane 3, 0.1 µg/mL; lane 4, 1 µg/mL; **B:** lane 1, 0 min; lane 2, 60 min; lane 3, 120 min; lane 4, 360 min. GIST-T1 cells were treated with SCF (10 ng/mL) for 1, 2, 6 h (C). **C:** lane 1, 0 h; lane 2, 1 h; lane 3, 2 h; lane 4, 6 h. GIST-T1 cells were treated with STI571 (1 µg/mL), LY294002 (50 µmol/L), PD98059 (50 µmol/L), or Wortmannin (100 nmol/L) for 6 h (D). **D:** Lane 1, non-treated; lane 2, STI571; lane 3, LY294002; lane 4, PD98059; lane 5, Wortmannin. 1×10^6 DLD-1 cells were treated with SCF in different concentrations for 6 h (E). **E:** Lane 1, non-treated; lane 2, SCF 5 ng/mL; lane 3, SCF 10 ng/mL; lane 4, SCF 15 ng/mL; lane 5, SCF 20 ng/mL; lane 6, SCF 100 ng/mL. GIST-T1 cells were treated with STI571, SCF, or combination of both for 6 h to determine the transcriptional level of HIF-1 alpha (F). **F:** Lane 1, non-treated; lane 2, STI571 1 µg/mL; lane 3, SCF 10 ng/mL; lane 4, SCF 10 ng/mL+STI571 1 µg/mL; lane 5, SCF 10 ng/mL+STI571 10 µg/mL.

formazan precipitate was solubilized by the addition of 100 µL of a 50% *N,N*-dimethyl formamide/10% SDS solution, and incubated for 4 h at room temperature. Sample absorbances were then measured on a plate reader at 570 nm.

Statistical analysis

Data were reported as mean \pm SD. Statistics were analyzed with the Student's *t*-test. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

Tyrosine phosphorylation of c-KIT

c-KIT in the non-treated cells was tyrosine phosphorylated (Figure 1, upper panel, lane 1) in the GIST-T1 cells. This phosphorylation was inhibited by STI571 treatment (Figure 1, upper panel lane 2). c-KIT protein is also shown by Western blotting (Figure 1, lower panel).

Transcription of VEGF and HIF-1 alpha

RT-PCR detected the transcription of VEGF or HIF-1 alpha in the GIST-T1 cells. In the GIST-T1 cells, VEGF121 and VEGF165 isoforms were detected (Figures 2A-2D). STI571 inhibited VEGF in a dose- and time-dependent manner (Figures 2A and 2B). On the contrary, STI571 could not inhibit HIF-1 alpha (Figure 2F, lane 2). SCF up-regulated VEGF (Figure 2C) but not HIF-1

alpha (Figure 2F, lane 3). PD98059, MEK inhibitor, did not inhibit the transcription of VEGF (Figure 2D, lane 4). LY294002 and Wortmannin, both the inhibitors of phosphatidylinositol 3-kinase (PI3K), also did not inhibit the transcription of VEGF in the GIST-T1 cells compared to STI571 (Figure 2D, lanes 3 and 5). SCF (10 ng/mL) upregulated the transcription of VEGF in the colon adenocarcinoma cell line, DLD-1 (Figure 2E, lane 3). However, in the case of high concentration of SCF stimulation (100 ng/mL for 6 h), transcriptions of VEGF were downregulated in the DLD-1 cells (Figure 2E, lane 6).

Intracellular expression of VEGF and HIF-1 alpha in the GIST-T1

Flow cytometry was used to show that VEGF or HIF-1 alpha was expressed in the non-treated GIST-T1 cells (Figures 3B and 4B). STI571 reduced the expression of VEGF (Figure 4C). SCF upregulated the expression of VEGF in the GIST-T1 cells (Figure 4D) and STI571 inhibited the upregulation of VEGF by SCF stimulation in the GIST-T1 cells (Figure 4E). HIF-1 alpha could not be influenced by STI571 treatment (Figure 3C). SCF also did not influence the expression of HIF-1 alpha (data not shown). Western blotting revealed that VEGF was expressed as a 21-ku band, which was downregulated by STI571 in a dose-dependent manner (Figure 5).

Effect of STI571 on GIST-T1 cell viability

Cell viability of the GIST-T1 cells was measured by MTT

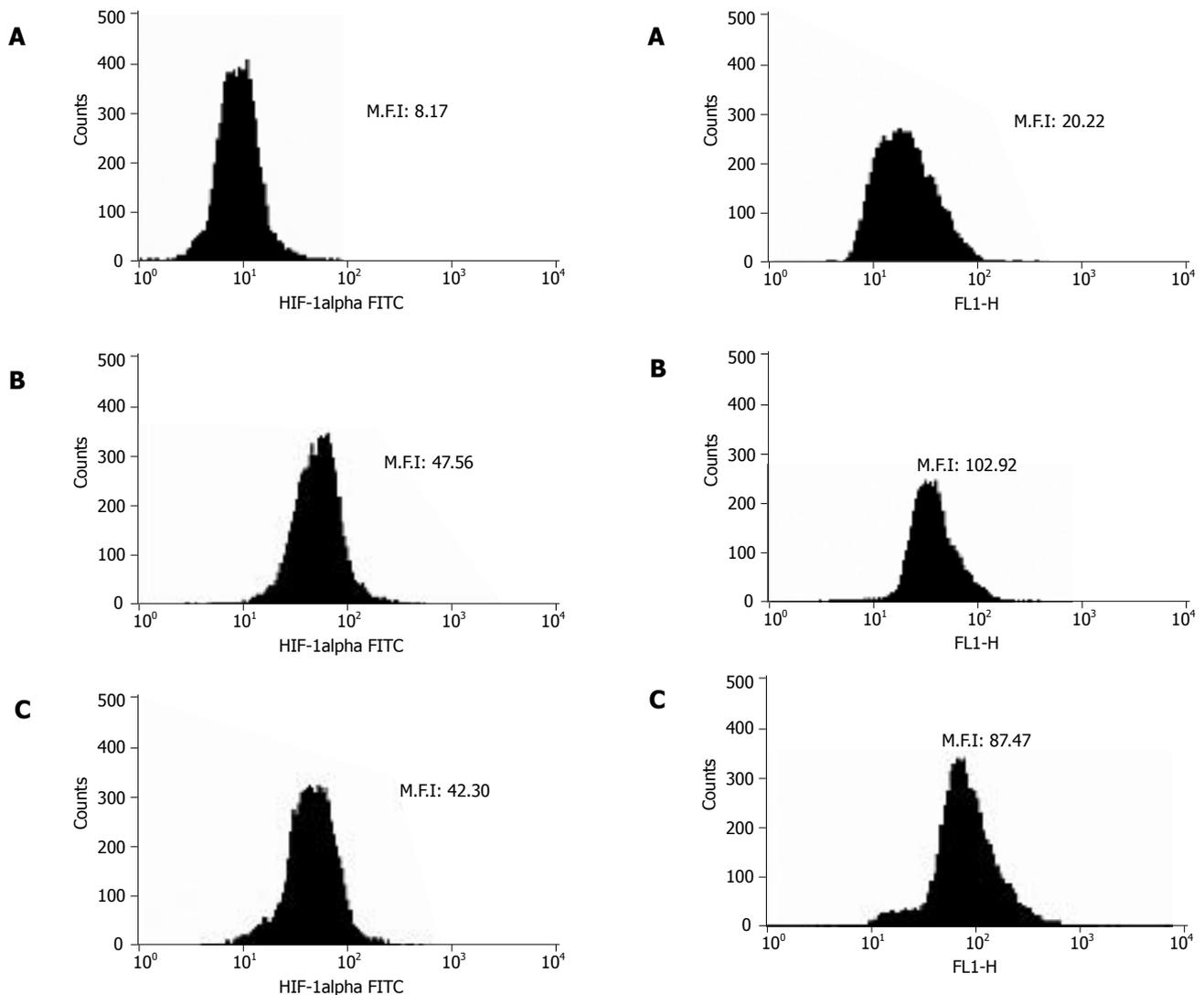


Figure 3 Expression of HIF-1 alpha was determined by flow cytometry. 2×10^6 GIST-T1 cells were treated with/without STI571 as described in "Materials and the methods. **A:** Control (normal rabbit IgG); **B:** non-treated (HIF-1 alpha antibody); **C:** STI571 (1 $\mu\text{g}/\text{mL}$). MFI, mean fluorescent intensity.

assay, which showed a reduction in viability following the treatment with STI571 to $37.9 \pm 1.3\%$, $36.5 \pm 1.7\%$, and $36.3 \pm 0.3\%$ at the concentrations of 0.1, 1, and 2 $\mu\text{g}/\text{mL}$, respectively ($P < 0.01$ vs non-treated). Cell viability in the case of non-treated cells was indicated as $100 \pm 0.5\%$.

DISCUSSION

The present study demonstrated that the recently developed compound STI571 inhibited the tyrosine phosphorylation of c-KIT in GIST-T1 tumor cells, as well as inhibiting both the transcription and translation of VEGF. Tyrosine phosphorylation of c-KIT was inhibited after 30 min treatment with STI571 at different concentrations (0.1 and 1 $\mu\text{g}/\text{mL}$), as assessed by Western blot analysis (data not shown). In contrast, Tuveson *et al*^[13] reported that tyrosine phosphorylation of another GIST cell line, GIST882, was not inhibited by a 1-h treatment with STI571 when used at 1 $\mu\text{mol}/\text{L}$ (0.59 $\mu\text{g}/\text{mL}$), as assessed by Western blot analysis. GIST882 has a

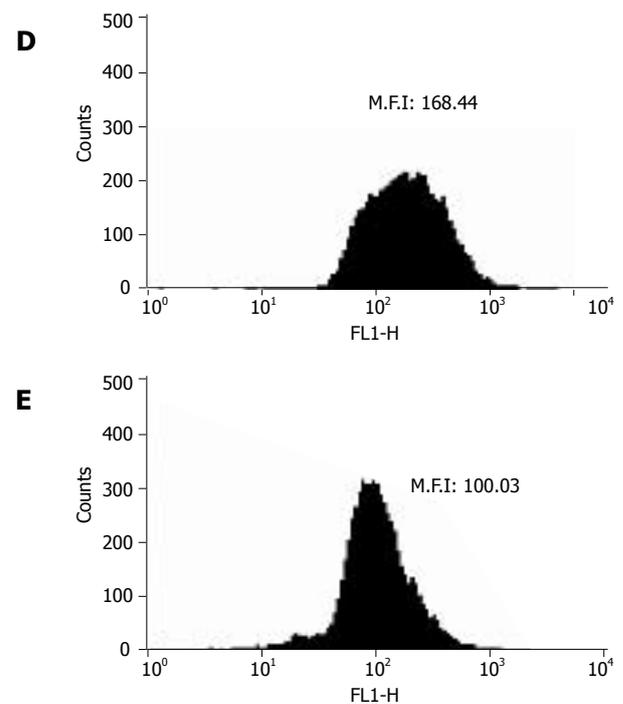


Figure 4 Expression of VEGF determined by flow cytometry. 2×10^6 GIST-T1 cells were treated with/without STI571 or SCF as described in "Materials and methods". **A:** Control (normal rabbit IgG); **B:** non-treated (VEGF antibody); **C:** STI571 (1 $\mu\text{g}/\text{mL}$); **D:** SCF (10 ng/mL); **E:** SCF (10 ng/mL)+STI571 (1 $\mu\text{g}/\text{mL}$). MFI, mean fluorescent intensity.

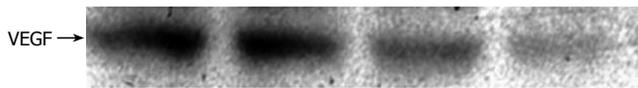


Figure 5 Assessment of expression of VEGF by Western blotting. 3×10^6 cells were treated with STI571 (0.01, 0.1, and 1 $\mu\text{g/mL}$) for 12 h, and then cells were rinsed with ice-cold PBS and lysed in RIPA buffer as described in "Materials and methods". Fifty micrograms of protein was applied in each lane. Lane 1: non-treated; lane 2: STI571 0.01 $\mu\text{g/mL}$; lane 3: STI571 0.1 $\mu\text{g/mL}$; lane 4: STI571 1 $\mu\text{g/mL}$.

homogenic mutation in the *c-kit* gene resulting in a K642E amino acid change and the constitutive activation of c-KIT^[15]. On the other hand, GIST-T1 has a heterogenic 57-bp deletion in exon 11 of the *c-kit* mutation, and the c-KIT protein in the GIST-T1 cells constitutively activated. Furthermore, Frolvo *et al*^[6] reported that STI571 inhibits the activation of p44/42 mitogen activated protein kinase (p44/42MAPK) in GIST882 cells, but we could not detect the same effect in our cells following STI571 treatment (data not shown). Oncogenic Ras family proteins activate the p44/42MAPK pathway, and this activation contributes to the increased proliferative rate of tumor cells^[17]. Phosphorylated c-KIT also activates the p44/42MAPK pathway as well as plays a role in the process of oncogenic activation^[18]. MEK is upstream of p44/42MAPK, and MEK inhibitors could inhibit the activation of p44/42MAPK. In our hands, the MEK inhibitor, PD98059, partially inhibited the activation of p44/42MAPK (data not shown). These results suggested that activation of p44/42MAPK was independent of the activation of c-KIT in GIST-T1 cells.

In a previous study, Ebos *et al*^[19] reported that STI571 reduced VEGF expression in the Bcr-Abl positive chronic myelogenous leukemia cells. They reported that activation of Bcr-Abl played an important role in VEGF expression. In our study, RT-PCR and flow cytometry analysis revealed that both the transcription and translation of VEGF in GIST-T1 cells were suppressed by STI571 treatment. Western blot analysis revealed that STI571 inhibited the expression of VEGF for 12-h incubation. SCF activated c-KIT and upregulated the expression of VEGF. These results suggested that activation of c-KIT was an important role in both the transcription and the translation of VEGF in the GIST-T1 cells. Furthermore we investigated the signal transduction cascade which was involved in the expression of VEGF in the GIST-T1 cells using MEK inhibitor or PI3K inhibitors. In our study, PD98059, LY294002 and Wortmannin had no such effect on these cells. LY294002 and Wortmannin are both inhibitors of PI3K and thereby inhibit activation of the AKT pathway^[20]. AKT promotes angiogenesis through eNOS activation^[20]. STI571 inhibited the activation of AKT in GIST882 cells^[6], but the same result was not obtained in our GIST-T1 cells by Western blotting (data not shown). These results suggested that neither the p44/42MAPK nor PI3K signaling pathways are involved in the regulation of VEGF expression in GIST-T1 cells.

Hypoxia inducible factor-1 (HIF-1) plays an important role in the expression of VEGF in the malignant tumors. HIF-1 activity is primarily regulated by the levels of

HIF-1 alpha in the cells^[21]. In our study, transcriptional or translational levels of HIF-1 alpha were not regulated by STI571 treatment or SCF stimulation.

c-KIT is expressed in the colon adenocarcinoma cell line, DLD-1^[22]. It is clear that c-KIT regulates the expression of VEGF in other cells. So, we observed whether c-KIT was involved in the upregulation of the transcriptional levels of VEGF using SCF in DLD-1 cells. SCF induced VEGF transcription at a concentration of 5–10 ng/mL. These results suggested that the expression of VEGF might be concerned with the activation of c-KIT.

MTT assay revealed that STI571 inhibited the cell viability of GIST-T1 cells. Activation of c-KIT in the GIST-T1 cells played an important role in cell survival signaling.

In conclusion, our results on the treatment of GIST-T1 cells with STI571 suggest that the expression of VEGF in these cells might be regulated via the c-KIT signal transduction cascade. STI571 inhibited not only cell viability but also the expression of VEGF in GIST-T1 cells, and it could be a useful compound for GIST therapy. Further studies are required to investigate the mechanism underlying VEGF regulation in more detail.

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Effect of increased hepatic platelet activating factor and its receptor portal hypertension in CCl₄-induced liver cirrhosis

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Abstract

AIM: To evaluate the changes in hepatic platelet activating factor (PAF) and its receptors and their effect on portal pressure of cirrhotic rats induced by CCl₄.

METHODS: A model of liver cirrhosis was replicated in rats by intra-peritoneal injection of CCl₄ for 8 wk. We determined the effect of hepatic PAF and its receptor level on portal and arterial pressure by EIA, saturation binding and RT-PCR technique.

RESULTS: Compared to control rats, cirrhotic rats had higher hepatic PAF levels and output as well as higher plasma PAF levels ($P < 0.01$, $P < 0.01$, $P < 0.05$, respectively). Both hepatic PAF receptor mRNA levels and PAF binding were nearly 3-fold greater in cirrhotic rats ($P < 0.01$). Portal injection of PAF (1 g/kg WT) increased the portal pressure by 22% and 33% in control and cirrhotic rats, respectively. In contrast, the arterial pressure was decreased in the both groups (54% in control rats and 42% in cirrhotic rats). Injection of the PAF antagonist BN52021 (5 mg/kg WT) decreased the portal pressure by 16% in cirrhotic rats but had no effect in the control rats.

CONCLUSION: The upregulation of the PAF system contributes to hepatic hemodynamic and metabolic abnormalities in cirrhosis, and the increased release of PAF into the circulation has impacts on the systemic hemodynamics.

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Key words: Platelet activating factor; PAF receptors; Endothelin; Portal hypertension; Cirrhosis

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INTRODUCTION

Platelet activating factor (PAF: 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is a phospholipid exhibiting diverse biological actions. PAF acts both as a multifunctional, soluble, proinflammatory agent and as a specific membrane-bound adhesion molecule. PAF receptors have been identified in smooth muscle cells, cardiomyocytes, endothelial cells, neutrophils, monocyte-macrophages, Kupffer cells and eosinophils^[1]. The physiological actions of PAF play a role in platelet secretion and aggregation, bronchoconstriction, vascular permeability and systemic arterial hypotension^[1-4]. Exogenous PAF administration via the portal vein increases portal venous pressure and activates glycogenolysis both in perfused organs^[5] and *in vivo*^[6]. In contrast, systemic infusion of PAF reduces arterial blood pressure^[7,8]. Portal hypertension and hyperdynamic systemic circulation are two prominent clinical features of human and animal experimental liver cirrhosis^[9-11]. Circulatory responses to intravenous infusion of PAF resemble the hemodynamic change in advanced liver cirrhosis. Excessive deposition of extracellular matrix is a cause of the increased resistance to hepatic blood flow and consequent portal hypertension in cirrhosis, but studies in animals indicate that increased vascular tone is also a contributing factor^[12].

The PAF content in intact liver is elevated by various types of injury, including ischemia-reperfusion^[13], obstructive jaundice^[14], and endotoxin exposure *in vivo*. Based on these observations, we hypothesized that increased hepatic synthesis of PAF in cirrhosis could lead to increased plasma levels, and that PAF might play a role in portal hypertension. Our results have confirmed that hepatic PAF levels are elevated and PAF receptors are upregulated in cirrhosis. The reactivity of the hepatic vasculature to PAF is consequently increased, while that of the systemic vasculature is attenuated.

MATERIALS AND METHODS

Induction of cirrhosis

Cirrhosis was induced in male Sprague-Dawley rats

weighing 230-250 g as described previously^[10,15,16]. Briefly, intraperitoneal injection of CCl₄ (0.15 mL/kg WT, twice a week for 8 wk) was combined with drinking water containing phenobarbital (0.4 g/L). Control rats received injection of the vehicle (peanut oil) and phenobarbital water.

Determination of portal venous and systemic arterial pressure

Rats were anesthetized with 50 mg/kg (i.p.) pentobarbital, and placed on a heated water blanket maintained at 38°C. A PE-50 catheter (Thomas Scientific, Swedesboro, NJ, USA) was inserted into the femoral artery to monitor arterial blood pressure via a strain gauge pressure transducer connected to a 4-channel Grass polygraph (Model no. 79E, Quincy, MA, USA). The hepatic trigone was exposed via laparotomy, and the portal vein was skeletonized. The intestines and the abdominal cavity were covered with warm saline-soaked sponges, and a catheter was inserted into the portal vein and connected to a pressure transducer coupled to a 4-channel Grass polygraph to monitor portal pressure. After stable recordings of both portal and arterial blood pressure were obtained, 1 mL of blood was withdrawn from the femoral artery, portal vein and suprahepatic vena cava, respectively. Then, the liver was rapidly excised and washed in ice-cold phosphate buffered saline containing 0.1 mmol/L EDTA and 0.1 mmol/L EGTA. A portion of the liver tissue was stored in 10% buffered formalin, a portion in OCT, and the rest was snap-frozen in liquid nitrogen and stored at -80 °C.

Morphometric analysis

An established histological grading system was employed for the determination of pathological scores of liver injury^[17-19]. Paraffin-embedded liver sections of 4- μ m thickness were stained with hematoxylin-eosin and Masson's trichrome. Steatosis, inflammation, necrosis and fibrosis were scored in at least three random fields of view in each tissue section and a score for each specific parameter was estimated. Steatosis was assessed by estimating the percentage of cells with micro- and macrovesicular fat as follows: 0 (absent), 1 (1-25%), 2 (26-50%), 3 (51-75%), and 4 (76-100%). Necrosis was scored as follows: 0 (absent), 1 (1-10 necrotic cells per view), 2 (11-20 necrotic cells per view), 3 (21-30 necrotic cells per view), 4 (31-40 necrotic cells per view), and 5 (41-50 necrotic cells per view). Inflammation was assessed as follows: 0 (absent), 1 (rare), 2 (scattered), 3 (scattered with localized foci), 4 (abundant with foci), and 5 (extensive). Architectural change, fibrosis and cirrhosis were estimated as follows: 0 (absent), 1 (rare), 2 (scattered deposition), 3 (scattered with localized deposition), 4 (abundant with minor bridging fibrosis), 5 (bridging fibrosis) and 6 (cirrhosis). A total pathology score was calculated by combining and summing the scores for the above pathological parameters, and the average score per animal/treatment group was calculated.

Determination of PAF in liver and blood

Lipids were extracted as previously described^[20]. Briefly, 100 mg of liver or 1 mL of blood was homogenized in 9.5 mL

of a mixture of methanol, chloroform and water (2:1:0.8, v:v:v). The homogenates were kept at room temperature for 1 h, followed by the addition of chloroform (2.5 mL) and water (2.5 mL). After being thoroughly mixed, the mixture was kept at room temperature for 1 h and centrifuged at 1200 r/min for 15 min. The chloroform layer was aspirated and dried under nitrogen at 35 °C. The residue was dissolved in 200 μ L chloroform, and applied to the Bond Elut SI column (Amprep silica mini-columns; Amersham Pharmacia Biotech, Piscataway, NJ, USA). The column was washed with 3 mL of chloroform, 2 mL of chloroform-methanol (6:4; v/v) and 3 mL of chloroform-methanol-28% aqueous ammonia (70:85:7, v:v:v). PAF was eluted with 2 mL of chloroform-methanol-28% aqueous ammonia (50:50:7, v/v). The elute was evaporated to dryness under nitrogen, and the residue was dissolved in 200 μ L of saline containing 0.01% Triton X-100. PAF concentration was determined by [³H]-PAF scintillation proximity assay (Amersham-Pharmacia Biotech).

Determination of hepatic PAF binding

Hepatic membranes were prepared as described previously^[10,16] and suspended in 50 mmol/L Tris-HCl (pH 8.0) containing 5 mmol/L MgCl₂, 125 mmol/L choline chloride, 0.1 mol/L PMSF, 0.1 μ g/mL leupeptin and 1 μ g/mL pepstatin. The membrane suspension was stored at -80 °C in aliquots after the protein concentration was adjusted to 10 mg protein/mL. Membranes (100 μ g protein) were incubated in 50 mmol/L Tris-HCl (pH 7.2) containing 5 mmol/L MgCl₂, 125 mmol/L choline chloride, 0.25% BSA and 0.125-32 nmol/L 1-O-[³H]octadecyl-2-acetyl-sn-glycero-3-phosphocholine (151 Ci/mmol, 9.96 GBq/mg; Amersham) \pm 10 μ mol/L unlabeled PAF (Bachem Americas, King of Prussia, PA, USA) at 30 °C for 1 h. The reaction was terminated with the addition of 5 mL ice-cold assay buffer and filtration through Whatman GF/C filters (Whatman, Hillsboro, OR, USA) presoaked in assay buffer for 1 h. Filters were washed thrice with 4 mL of assay buffer, and the radioactivity was determined in a β -scintillation counter.

Determination of hepatic PAF receptor mRNA levels

The mRNA expression of PAF receptors was determined by semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR). RNA was isolated from the livers using a RNA isolation kit (ToTALLY RNATM, Ambion, Austin, TX, USA). Two micrograms of total RNA was used for the preparation of cDNA by reverse transcription as described. cDNA equivalent of 5 ng original RNA was used in PCR. The reaction mixture (50 μ L) contained 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 0.2 mmol/L dNTPs, 20 pmol of PCR primers and 2U platinum Taq DNA polymerase (GIBCO-Invitrogen, Carlsbad, CA, USA). Thirty-five cycles of reaction were carried out as follows: denaturation at 94 °C for 1 min, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. The PCR primers used for PAF cDNA were: 5'-GCCACAACAGAGGCTTGA-3'(forward) and 5'-TC CATTGCTCTGGGCAGGAA-3'(reverse) [product size, 121 bp^[21]]. For normalization, β -actin mRNA levels were measured as described previously^[15], using cDNA primers:

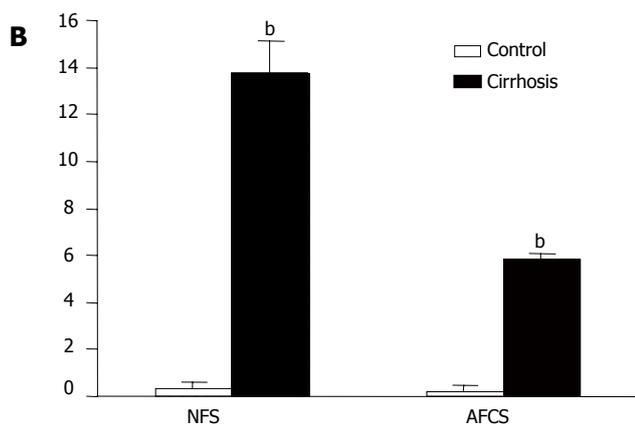
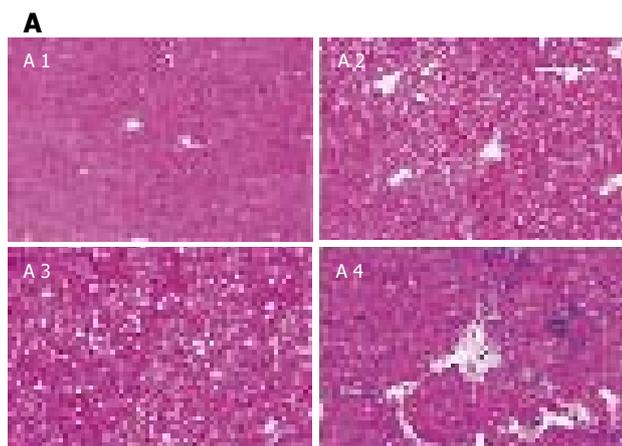


Figure 1 Morphometric analysis of cirrhotic liver 8 wk after CCl₄ or vehicle treatment (A) and scores for necroinflammatory NFS, architectural change, fibrosis and cirrhosis (AFCS) (B). ^b*P* < 0.01 vs control.

| | Control | Cirrhotic rats | <i>P</i> |
|-----------------------------|-------------|----------------|----------|
| Liver/body weight ratio (%) | 4.80 ± 0.25 | 4.90 ± 0.44 | NS |
| Weight of spleen (g) | 0.73 ± 0.06 | 1.36 ± 0.15 | 0.001 |
| Portal pressure (kPa) | 0.71 ± 0.08 | 1.63 ± 0.09 | 0.001 |
| MAP (kPa) | 15.2 ± 1.2 | 10.9 ± 1.3 | 0.01 |
| PAF (ng/g) | 2.74 ± 0.49 | 3.95 ± 0.43 | 0.01 |

NS: not significant.

5'-TTCTACAATGAGCTGCGTGTG-3' (forward) and 5'-TTCATGGATGCCACAGGATTC-3' (reverse) [product size, 561 bp^[22]]. The PCR products were resolved in a 25 g/L agarose gel and stained with SYBR Green I (FMC Bioproduct, Rockland, ME, USA). The gels were scanned under blue fluorescent light using a phosphorimager and the band intensity was quantified using ImageQuANT software (Molecular Dynamics, Sunnyvale, CA, USA).

Determination of PAF effect on portal venous and systemic arterial pressure

The experiment was essentially conducted for the measurement of baseline pressure. After a stable recording of portal and systemic mean arterial pressure (MAP) was

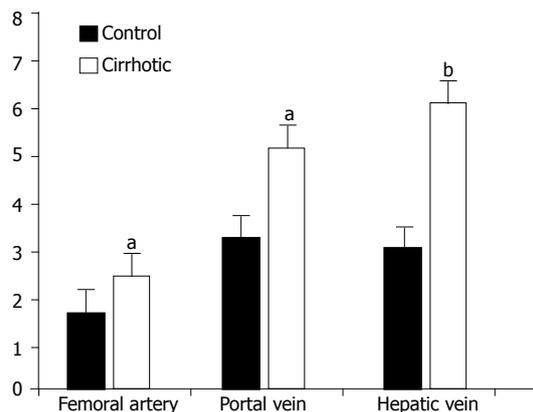


Figure 2 Effect of cirrhosis on circulating PAF levels. Concentrations of PAF in blood from femoral artery, portal and hepatic vein (suprahepatic vena cava) were determined. Values are mean ± SD. ^a*P* < 0.05 vs control; ^b*P* < 0.01 vs control.

obtained, 1 mL of a solution containing PAF (1 μg/kg) was infused over 1 min from a 975 Harvard apparatus compact infusion pump into the portal vein via a 23 gauge needle/PE50 catheter. Portal venous pressure and MAP were monitored continuously for 15 min.

Statistical analysis

The values were presented as mean ± SD. Student's *t*-test was employed for statistical comparison between the groups. *P* < 0.05 was considered statistically significant.

RESULTS

Characteristics of cirrhosis

There were no notable changes in liver histology throughout the 8 wk in control rats (Figure 1A1). In contrast, CCl₄-treated rats demonstrated extensive changes in liver morphology, including steatosis, inflammation, hepatocyte ballooning, and necrosis. Extensively distorted architecture due to excessive deposition of extracellular matrix was observed, which caused bridging of fibrosis, and infiltration of inflammatory cells in sinusoids and their accumulation around the islands of hepatocytes (Figure 1A3). Hepatic fibrosis was further validated histologically with Masson's trichrome staining (Figures 1A2 and A4).

The liver/body weight ratio was not significantly different between control and cirrhotic rats, whereas spleen weight of cirrhotic rats was nearly doubled (Table 1). MAP was significantly lower in cirrhotic rats (10.9 ± 1.3 kPa) than in control rats (15.2 ± 1.2 kPa). Portal venous pressure was higher in cirrhotic rats (1.63 ± 0.09 kPa) than in control rats (0.71 ± 0.08 kPa), indicating portal hypertension (Table 1).

Effect of cirrhosis on hepatic and circulating PAF and hepatic PAF receptor levels

Hepatic PAF concentrations were increased by 44% in cirrhotic rats (Table 1). Concentrations of PAF in the femoral arterial, portal venous, and hepatic arterial blood were significantly greater in cirrhotic rats than in control rats (Figure 2). Moreover, the concentration of PAF was significantly greater in hepatic venous blood than in

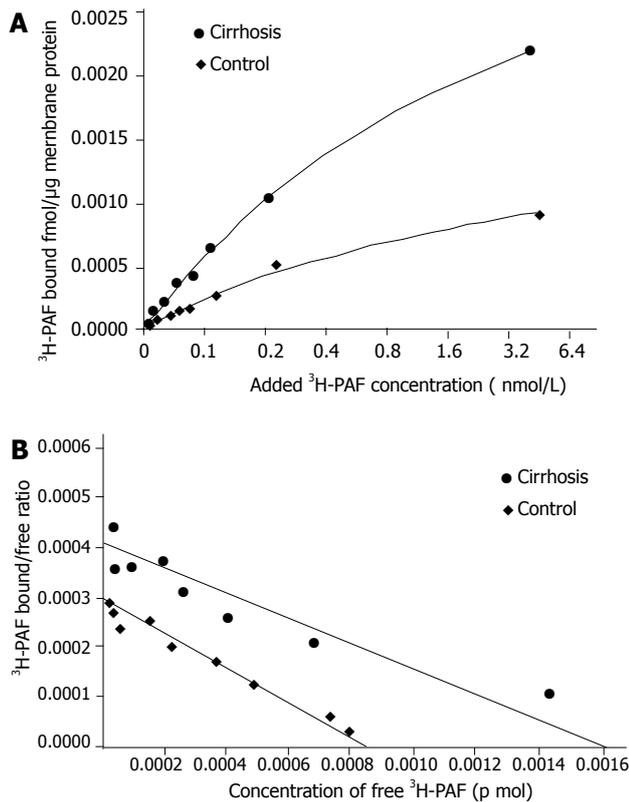


Figure 3 Effect of cirrhosis on hepatic PAF binding. **A:** Results of the saturation binding assay. ³H-PAF was incubated with 100 μg membrane protein, in the presence or absence of 10 μmol/L at 30 °C for 1 h; **B:** Scatchard plot analysis of ³H-PAF binding to hepatic tissue of cirrhotic and control rats. Values are mean of separate experiments. Cirrhosis: $R=0.98$, $K_d=8.013$ nmol/L, $B_{max}=2.8\pm 0.213$ fmol/μg membrane protein; control: $R=0.99$, $K_d=5.794$ nmol/L, $B_{max}=0.9\pm 0.061$ fmol/μg membrane protein.

portal venous blood in cirrhotic rats (Figure 2). No such difference in hepatic venous and portal venous blood of control rats was observed.

Scatchard analysis of the saturation binding data revealed a 3-fold increase in PAF binding capacity in cirrhotic liver as compared to the control (B_{max} of 2.8 ± 0.21 vs 0.9 ± 0.06 fmol/μg protein, $P<0.01$), whereas receptor affinity was unaltered (K_d : 8.01 ± 1.33 nmol/L for cirrhotic rats and 5.79 ± 0.96 nmol/L for control rats, $P<0.05$, Figure 3). Consistent with the increased receptor density, a similar increase in the PAF receptor mRNA transcript was observed in the cirrhotic liver as determined by RT-PCR (PAFR/ β -actin mRNA ratio of 0.51 ± 0.03 vs 0.16 ± 0.015 , $P<0.01$, Figure 4).

Effect of PAF on portal and arterial pressure in cirrhotic rats

A separate set of control and cirrhotic rats were used to determine the PAF-induced changes in portal and systemic blood pressure. Infusion of PAF (1 g/kg WT) via the portal vein after a short delay caused a greater increase ($P<0.01$) in portal pressure in cirrhotic rats (from 1.61 ± 0.08 to 2.13 ± 0.09 kPa; 33% increase) than in control rats (from 1.07 ± 0.10 to 1.30 ± 0.09 kPa; 22% increase) (Figures 5 and 6). Following the initial rise, the portal pressure decreased progressively with time and was lower than the basal value 9 min after the administration

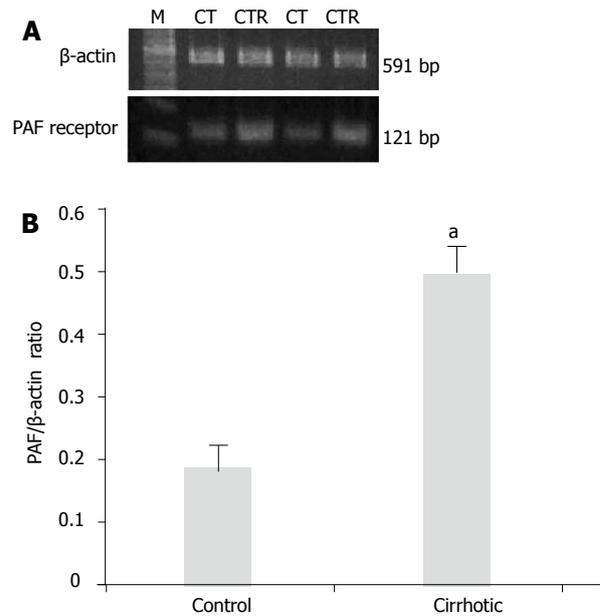


Figure 4 Effect of cirrhosis on mRNA expression of hepatic PAF receptor. **A:** PCR products of PAF and β -actin from control (CT) and cirrhotic (CR) rat livers; **B:** ratio of the PAF receptor and β -actin mRNA from six samples. $^aP<0.01$ vs control.

of PAF.

The effect of PAF administered into the portal vein on arterial pressure was opposite to that observed on portal pressure (Figures 5 and 6). In contrast to the delay onset of the rise in portal venous pressure, MAP fell immediately after the administration of PAF. The decrease in MAP ($P<0.01$) was greater in control rats (54%) than in cirrhotic rats (42%). With the fall in MAP, pulse pressure fell markedly and did not recover, suggesting a reduction in cardiac contractility.

Effect of PAF antagonist BN52021 on portal and arterial pressure in cirrhotic rats

Administration of BN52021 (5 mg/kg WT) via the portal vein caused a 16% decrease in the portal pressure of cirrhotic rats (from 1.95 ± 0.21 to 1.64 ± 0.15 kPa, $P<0.01$). This effect occurred 2 to 3 min after the administration of BN52021. On the other hand, arterial pressure did not change upon treatment with BN52021 (from 11.0 ± 0.8 to 11.3 ± 0.67 kPa). In control rats, BN52021 had no effect on either portal or arterial pressure (Figure 7).

DISCUSSION

This investigation showed that increased production of PAF in the cirrhotic liver could lead to an increased circulating level of PAF, which is likely responsible for the arterial hypotension associated with the disease. In addition, hepatic PAF receptor mRNA expression was upregulated with a concomitant increase in PAF binding. Since PAF potently increases portal pressure and hepatic vascular resistance^[6,7,23], it can be concluded that the up-regulated PAF system in the liver plays an important role in portal hypertension.

In the present study, the source of the increased hepatic PAF and the site of increased PAF receptors were not

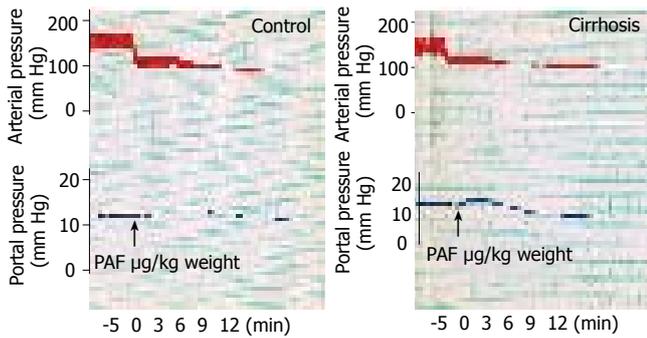


Figure 5 Tracings of the femoral arterial and portal venous pressure. After stabilization of arterial and portal venous pressure, PAF (1 µg/kg WT) was infused and the pressure was recorded continuously.

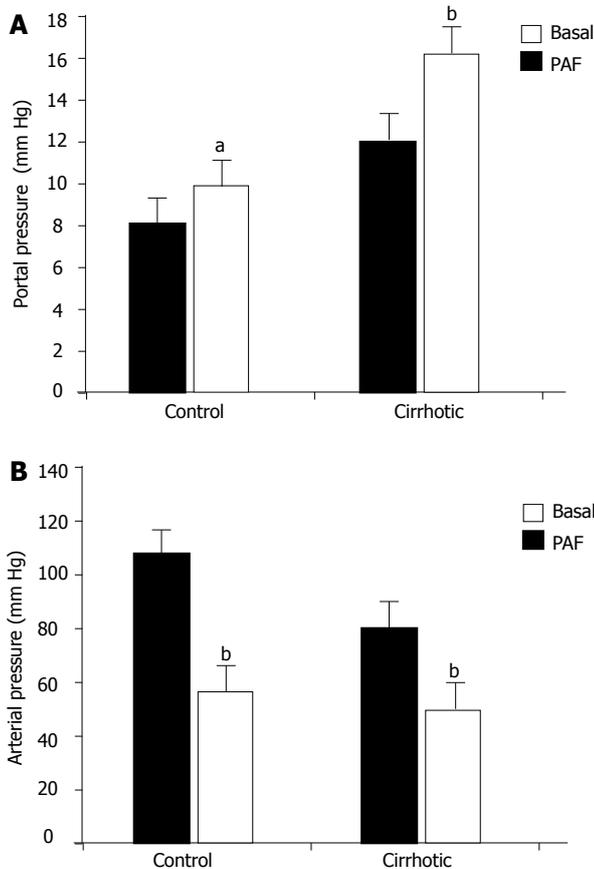


Figure 6 Effect of cirrhosis on PAF-induced changes in arterial and portal pressure. Arterial and portal venous pressure before and after portal administration of PAF were measured. Values are mean±SD of six determinations. ^a*P*<0.05 vs basal value; ^b*P*<0.01 vs basal value.

determined. Yang *et al*^[10] reported that Kupffer cells are a major source of increased PAF in CCl₄-induced cirrhotic rat liver. Synthesis of PAF by Kupffer cells is stimulated by a number of inflammatory mediators and bacterial endotoxin (ET)^[24,25]. Increased circulating ET is a strong candidate contributor to the elevation of hepatic portal pressure in cirrhosis^[15,26]. We have previously reported that hepatic ET-1 and its receptors increase in rats 24 h after the administration of CCl₄^[15] and progressively thereafter during the development of cirrhosis. In the present study, hepatic PAF did not increase during the first

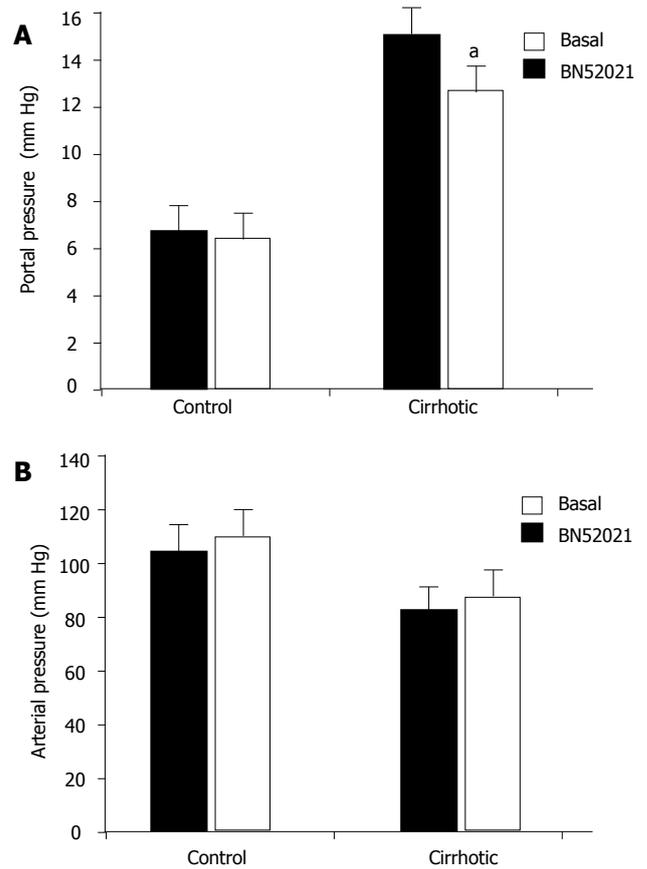


Figure 7 Effect of BN52021 on femoral arterial and portal venous pressure of cirrhotic rats 8 wk after vehicle or CCl₄ treatment. Values are mean±SD 5-6 min after introduction of BN52021. ^a*P*<0.05 vs basal value.

week of CCl₄ treatment (2.70±0.75 ng/g in control rats and 2.74±0.46 ng/g in CCl₄-treated rats, *P*>0.05) but increased after 2 wk of treatment. Since PAF synthesis by Kupffer cells is increased by ET-1^[24], the early elevation of ET-1 may contribute to the long-term increase in hepatic PAF. To further investigate the interaction between these two potent mediators during cirrhosis, we infused the powerful vasoconstrictor ET-1 into some experimental animals. After stabilization of the portal and arterial pressures following the administration of PAF, ET-1 (0.75 nmol) was infused as a bolus into the portal vein. The magnitude of ET-1 effect was much greater in control rats (200% increase) than in cirrhotic rats (80% increase, *P*<0.01). We have previously reported that portal infusion of ET-1 alone causes a 150% increase in portal pressure in control rats and a 23% increase in cirrhotic rats^[15], suggesting that with or without prior infusion of PAF, the qualitative responses to ET-1 are similar between control and cirrhotic rats. Quantitatively, prior infusion of PAF appears to sensitize the hepatic vasculature to ET-1 moderately in control rats (200% vs 150%) and greatly in cirrhotic rats (80% vs 23%). The interaction between these two potent mediators during cirrhosis warrants further investigation.

Potentially, PAF could be increased by a decrease in the activity of plasma PAF-acetylhydrolase, which is predominantly responsible for the hydrolysis of PAF to lyso-PAF^[27,28]. Plasma PAF-acetylhydrolase activity is similar

in normal individuals and patients with alcohol-induced liver cirrhosis^[29]. Patients with primary and secondary biliary cirrhosis have elevated levels of circulating PAF and serum PAF-acetylhydrolase activity^[30], suggesting that synthesis of PAF increased by plasma PAF-acetylhydrolase is the mechanism underlying the elevated circulating levels of PAF.

The augmented effect of PAF on the vasculature of chronically injured liver appears to be independent of the hemodynamic state of the liver (i.e. normotensive or portal hypertensive). The vasoconstrictor effect of PAF in the perfused livers is greater in rats with thioacetamide-induced hepatic fibrosis than in control rats, even though the fibrosis rats have no portal hypertension^[31]. In the present study, PAF-induced portal hypertension was of greater magnitude in the cirrhotic rats than in the control rats. These results suggest that the increased reactivity to PAF is associated with the pathology of hepatic cirrhosis or injury rather than the pre-existing presence or absence of portal hypertension. That is to say, the response to PAF is not dependent upon the already increased hepatic vascular tone.

The reduction of portal pressure in cirrhotic rats induced by the PAF antagonist BN52021 provides convincing evidence that endogenous PAF is involved in the development of portal hypertension. The mechanisms of PAF-induced hepatic vasoconstriction are very complex and remain to be defined. PAF receptors have been found in smooth muscle cells^[32]. PAF elevates cytosolic free calcium in smooth cells^[33,34], which is necessary for muscle contraction. Exogenous PAF causes contraction of smooth muscle from ileal and peripheral lung strips^[35,36] and contributes to endothelin-induced vascular constriction in rat mesentery^[37]. Therefore, the contractile effect of PAF on the hepatic vasculature may be elicited by its direct action on smooth muscle cells. PAF may also act by stimulating the synthesis of eicosanoids such as thromboxane, PGE₂, and PGD₂, which are known to cause portal vasoconstriction^[38-41].

The combination of portal hypertension and peripheral vasodilatation with systemic arterial hypotension is characteristic of liver cirrhosis and implicates PAF. The observation that PAF induces smooth muscle contraction of ileal^[35] and pulmonary strips^[36], and micro-vessels^[37] is apparently in contradiction with the observation that it induces hypotension when administered intravenously to rats^[42]. In fact, PAF produces hypotension in all animal species studied^[8]. Increased plasma levels of PAF in cirrhosis are associated with low peripheral vascular resistance that is reversed by PAF antagonist BN52021^[9,29,43]. Sakaguchi *et al*^[44] and Hines *et al*^[6] reported that intravenous administration of PAF (1.5 µg/kg WT) causes arterial hypotension. The mechanisms of PAF-induced systemic arterial hypotension are not clearly understood. PAF-induced hypotension is not mediated by the central nervous system, renin-angiotensin system, β-adrenergic and dopaminergic eicosanoids, as well as Ca²⁺ influx and thyrotropin releasing steroids^[45,46]. However, PAF-induced delay and persistent hypotension is inhibited by the nitric oxide synthase inhibitor, N^ω-nitro-L-arginine^[47] while nitric oxide plays a role in PAF-induced relaxation

of rat thoracic aorta^[48]. This can be partially explained by the observation that PAF released by the liver may regulate systemic hemodynamics or stimulate the release of very powerful hypotensive agents such as nitric oxide^[47,48]. The involvement of nitric oxide requires clarification.

In conclusion, PAF is an important mediator of hepatic pathology during chronic liver injury. Cirrhotic liver is a source of circulating PAF and a major contributor to the systemic hypotension associated with liver cirrhosis.

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BASIC RESEARCH

IL-1 β activates p44/42 and p38 mitogen-activated protein kinases via different pathways in cat esophageal smooth muscle cells

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Abstract

AIM: To examine the pathway related to the IL-1 β -induced activation of mitogen-activated protein (MAP) kinases in cat esophageal smooth muscle cells.

METHODS: Culture of the esophageal smooth muscle cells from cat was prepared. Specific inhibitors were treated before applying the IL-1 β . Western blot analysis was performed to detect the expressions of COX, iNOS and MAP kinases.

RESULTS: In the primary cultured cells, although IL-1 β failed to upregulate the COX and iNOS levels, the levels of the phosphorylated forms of p44/42 MAP kinase and p38 MAP kinase increased in both concentration- and time-dependent manner, of which the level of activation reached a maximum within 3 and 18 h, respectively. The pertussis toxin reduced the level of p44/42 MAP kinase phosphorylation. Tyrphostin 51 and genistein also inhibited this activation. Neomycin decreased the density of the p44/42 MAP kinase band to the basal level. Phosphokinase C (PKC) was found to play a mediating role in the IL-1 β -induced p44/42 MAP kinase activity. In contrast, the activation of p38 MAP kinase was inhibited only by a pretreatment with forskolin, and was unaffected by the other compounds.

CONCLUSION: Based on these results, IL-1 β -induced p44/42 MAP kinase activation is mediated by the Gi protein, tyrosine kinase, phospholipase C (PLC) and PKC. The pathway for p38 MAP kinase phosphorylation is different from that of p44/42 MAP kinase, suggesting that it plays a different role in the cellular response to IL-1 β .

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INTRODUCTION

Esophageal reflux is a common condition that affects both children and adults. If left untreated, it can result in chronic esophagitis, aspiration pneumonia, esophageal strictures and Barrett's esophagus, which is a premalignant condition^[1]. Reflux esophagitis (RE) is a multifactorial disease that may depend on the relaxation of the transient lower esophageal sphincter (LES), the speed of esophageal clearance, the mucosal resistance and other factors, and is often associated with the LES pressure^[2]. Cytokines exhibit potent chemotactic activity toward different populations of leukocytes, and are essentially involved in the induction of acute and chronic inflammatory reactions^[3,4]. The selective infiltration of various leukocyte subsets into the esophagus indicates the participation of chemokines in the immune and inflammatory processes of RE.

Interleukin-1 (IL-1) has several biological effects on many cell types, and plays a key role in regulating the inflammatory reaction^[5]. The IL-1 family is an important part of the innate immune system, which regulates functions of the adaptive immune system. In the presence of excess IL-1, inflammatory and autoimmune diseases can develop in many organs, including the joints, lungs, gastrointestinal track, central nervous system, or blood vessels. Stimulation of the cells by IL-1 initiates the transcription of many pro-inflammatory genes, including IL-6 and IL-8. The levels of the inflammatory genes are higher in the esophageal mucosa of those patients with RE^[6]. IL-1 has its own identical biological functions and it is induced by gastric ulceration in rats^[7,8]. Increased mucosal levels of IL-1 are constantly observed during acute and chronic intestinal inflammation in human beings^[9,10] and animals^[11,12]. The immunoneutralization of the IL-1 activity has been reported to greatly reduce the severity of colitis in a murine model of the disease,

suggesting that this cytokine plays an important role in initiating inflammation^[12]. The IL-1 mRNA and protein were strongly expressed in the gastric mucosa of gastritis and ulcer patients^[13,14]. Therefore, it is suggested that the pro-inflammatory effects of IL-1 are due to the induction of COX-2 gene expression in many tissues, including mucous^[14]. However, there are few reports about the effect on the smooth muscle.

In mammalian cells, there have been at least three different subfamilies of mitogen-activated protein (MAP) kinases reported. They include the extracellular signal-regulated kinases (ERKs, p44/42 MAP kinase), *c*-Jun N-terminal kinase (JNK), and p38. These kinases are activated by distinct upstream MAP kinases (MEKs), which phosphorylate the residues within a tripeptide motif (Thr-X-Tyr) on the MAP. Once activated, MAP kinases, in turn, phosphorylate a variety of intracellular substrates, including certain transcription factors^[15]. IL-1 β is known to activate all three MAP kinase subfamilies in human airway smooth muscle cells. IL-1 β stimulation has been associated with several MAP kinases cascades involved in the transmission of a signal from the cell surface to the nucleus. Although these pathways have not been demonstrated to be involved in the IL-1 β -mediated effects on the esophageal smooth muscle, p38 and ERK have been shown to contribute to such effects in human^[16] or canine airway^[17] or myometrial^[18] smooth muscle. Therefore, in this study, we examined the mechanism associated with IL-1 β -induced MAP kinases activation in cat esophageal smooth muscle cells.

MATERIALS AND METHODS

Materials

Fetal bovine serum (FBS) was purchased from Biofluids (Rockville, MD, USA). Dulbecco's modified Eagle's medium (DMEM), antibiotic-antimycotic (penicillin, streptomycin, amphotericin B), and trypsin-EDTA were obtained from Invitrogen (Grand Island, NY, USA). Phospho-p44/p42 monoclonal MAP kinase antibody, phospho-SAPK/JNK monoclonal antibody, phospho-p38 monoclonal MAP kinase antibody, p44/42 MAP kinase antibody, SAPK/JNK antibody and p38 MAP kinase antibody were acquired from Cell Signaling (Beverly, MA, USA). Goat anti-mouse IgG-HRP was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The rainbow prestained molecular weight marker was obtained from Amersham (Arlington Heights, IL, USA). The enhanced chemiluminescence agents were purchased from PerkinElmer Life Sciences (Boston, MA, USA). Sodium dodecyl sulfate (SDS) sample buffer was acquired from Owl Scientific, Inc. (Woburn, MA, USA). Nitrocellulose membrane, Tris/glycine/SDS buffer and Tris/glycine buffer were purchased from BioRad (Richmond, CA, USA). Phosphate-buffered saline (PBS) was acquired from Boehringer Mannheim (Indianapolis, IN, USA). The PD 98059 and SB202190 were obtained from Calbiochem (La Jolla, CA, USA). The GF 109203X was purchased from Tocris (Ellisville, MO, USA). RestoreTM Western Blot Stripping Buffer was obtained from Pierce (Rockford, IL, USA). Horseradish peroxidase-conjugated goat anti-

rabbit antibody, phorbol-12-myristate-13-acetate (PMA), genistein, tyrphostin 51 and other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Preparation of feline esophageal smooth muscle tissue and cell culture

Adult cats of either gender, weighing between 2.5 and 3.5 kg, were used in this study. The cats were anesthetized with ketamine (50 mg/mL per kg) and the abdomen was then opened with a midline incision. The esophagus and stomach were excised together, cleaned and maintained in Krebs buffer with the following composition (mmol/L): NaCl 116.6, NaHCO₃ 21.9, NaH₂PO₄ 1.2, KCl 3.4, CaCl₂ 2.5, glucose 5.4 and MgCl₂ 1.2. The esophagus and stomach were opened along the lesser curvature. The location of the squamocolumnar junction was identified and the mucosa was peeled off. The high-pressure zone was identified by a visible thickening of the circular muscle layer in conjunction with the squamocolumnar junction and immediately proximal to the sling fibers of the stomach. It has been shown that a 5- to 8-mm band of tissue coinciding with the thickened area constitutes the LES, and has distinct *in vivo* characteristics in an organ bath or as single cells after enzymatic digestion^[19,20].

After opening the esophagus and stomach and identifying the LES, the mucosa and submucosal connective tissues were removed via a sharp dissection. The LES was excised and a 3- to 5-mm wide strip at the junction of the LES and esophagus was discarded in order to avoid potential overlap. The circular muscle layer from the esophagus was cut into 0.5-mm-thick slices using a Stadie Riggs tissue slicer (Tomas Scientific Apparatus, Philadelphia, PA, USA). The last slices containing the myenteric plexus, the longitudinal muscle and the serosa were discarded, and the remaining slices were then cut into 2 mm \times 2 mm tissue squares by hand.

The sliced tissue was then placed into DMEM supplemented with 500 mL/L FBS containing 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 0.25 μ g/mL amphotericin B and incubated at 37 $^{\circ}$ C in a humidified atmosphere containing 50 mL/L CO₂ and 950 mL/L air. On the following day, fresh DMEM containing 100 mL/L FBS was added. Ten days later, the tissue explants were removed and the medium was exchanged with fresh DMEM containing 100 mL/L FBS. After the cells reached confluence, the cells were detached with 10 g/L trypsin-EDTA in HBSS with sodium bicarbonate. The cells were then counted, seeded at 1×10^6 cells/mL on 100-mm culture dishes, and maintained in DMEM containing 100 mL/L FBS. The medium was changed every 48 h until the cells reached confluence. The experiments were performed on the cells of passage 2.

In order to characterize the isolated and cultured esophageal smooth muscle cells as well as to exclude contamination by epithelial cells and fibroblasts, the cells were identified using an indirect immunofluorescent staining method with a monoclonal antibody of a light chain myosin^[21]. More than 95% of the cell preparation was found to be composed of smooth muscle cells.

Preparation of cell extracts and Western blot analysis

For the animal esophagitis model, esophageal smooth

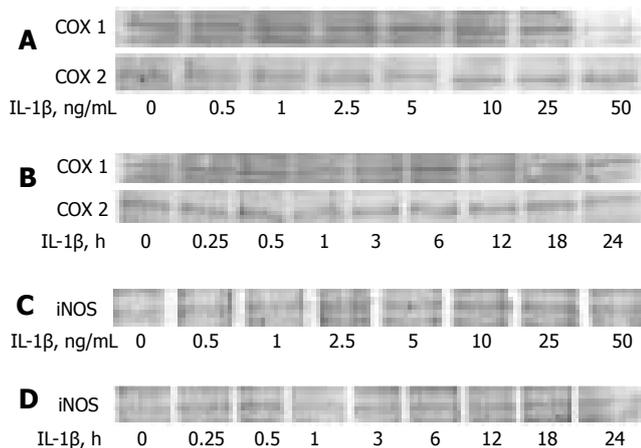


Figure 1 No increase of COX and iNOS expression levels by IL-1 β . (A) The cultured cat ESMCs were treated with IL-1 β for 18 h at each dose scale. The COX-1 and COX-2 expression levels were not altered by IL-1 β at concentrations up to 50 ng/mL. (B) The IL-1 β (25 ng/mL) treatment also failed to elevate the level after 24 h. (C) iNOS expression was unaffected by IL-1 β .

muscle tissue squares were homogenized in an homogenizing buffer containing 20 mmol/L Tris (hydroxymethyl) aminomethane (Tris), 160 mmol/L HCl (pH 7.5), 0.5 mmol/L EGTA, 0.5 mmol/L EDTA, 10 μ g/mL leupeptin, 10 μ g/mL aprotinin, 10 mmol/L β -mercaptoethanol and centrifuged at 14000 g for 15 min at 4 $^{\circ}$ C. The supernatants were used as the whole cell extract.

For the IL-1 β treatment experiments, the cells were plated in six-well culture plates and made quiescent at confluence by incubating them in serum-free DMEM for 24 h. The growth-arrested esophageal smooth muscle cells were incubated with or without IL-1 β at 37 $^{\circ}$ C at various times. When the inhibitors were used, they were added 1 h before applying the IL-1 β according to the inhibitors, with the exception that the cells were pretreated with the pertussis toxin 24 h prior to IL-1 β . After incubation, the cells were rapidly washed with ice-cold PBS and lysed for 5 min in an ice-cold lysis buffer containing 25 mmol/L Tris-HCl (pH 7.4), 25 mmol/L NaCl, 25 mmol/L NaF, 25 mmol/L sodium pyrophosphate, 1 mmol/L sodium vanadate, 2.5 mmol/L EDTA, 2.5 mmol/L EGTA, 0.5 g/L Triton X-100, 5 g/L SDS, 0.5 g/L deoxycholate, 0.5 g/L NP-40, 5 μ g/mL leupeptin, 5 μ g/mL aprotinin, and 1 mmol/L PMSF. After incubation, the lysates were scrapped and collected by centrifugation at 45000 g for 1 h at 4 $^{\circ}$ C to yield the whole cell extract.

Equal amounts of the protein from each sample were resolved on a SDS-polyacrylamide gel by electrophoresis. Prestained molecular mass markers were also run in an adjacent lane to determine the molecular mass. The separated proteins were transferred to a 0.45- μ m nitrocellulose membrane in 25 mmol/L Tris (pH 8.3), 192 mmol/L glycine, and 100 mL/L methanol using a power supply (Power Pac 1000, BioRad, Melville, NY, USA). The membranes were incubated in a PBS buffer containing 50 g/L non-fat dry milk for 1 h at room temperature in order to block the nonspecific binding. After washing thrice with PBS, the blots were incubated

with the primary antibodies in a PBS solution containing 1 g/L BSA at 4 $^{\circ}$ C overnight. The membranes were washed using PBS containing 0.5 g/L Tween 20 and then incubated with the horseradish peroxidase-conjugated secondary antibody (1:1000 dilution) for 1 h. The immunoreactive bands were visualized by enhanced chemiluminescence (ECL; Amersham). The total MAP kinase expression level was determined by subsequently stripping the same blot with a stripping buffer and reprobing the blot with p44/42 MAP kinase antibodies. Developed films from ECL were scanned and analyzed densitometrically using Scion Image.

Protein determination

The protein concentration of the supernatant in each reaction vial was measured spectrophotometrically at a wavelength of 595 nm using a BioRad assay (BioRad Chemical Division, Richmond, CA, USA).

Statistical analysis

The data were expressed as mean \pm SE of three separate experiments, and the statistical differences between means were determined by using Student's t test. A $P < 0.05$ was considered statistically significant.

RESULTS

The treatment of the esophageal smooth muscle cells (ESMC) with IL-1 β did not increase the COX-2 expression level (Figure 1). In other cell types such as canine tracheal smooth muscle cells^[22], human gastric cancer cells^[23], and human myometrial smooth muscle cells^[18], it has been reported that IL-1 β exerts its biological effects partly by inducing COX-2 expression. However, the cells treated with IL-1 β at a concentration of 0.5-50 ng/mL for 18 h did not show an increased COX-1 and COX-2 expression levels (Figure 1A). In addition, when treated with 25 ng/mL IL-1 β for 15 min to 24 h, the COX expression level in the cat ESMC was not altered (Figure 1B). These results corresponded to previous results showing that an experimentally induced rat esophagitis did not cause an increase in the COX-2 level, suggesting that COX-2 might not mediate esophagitis (unpublished data).

It is also known that IL-1 β mediates inflammation, at least in part, by inducing the expression of inducible nitric oxide synthase (iNOS)^[7], with the concomitant enhanced synthesis of NO, which is a critical effector molecule, in rat pulmonary artery smooth muscle cells. The cat ESMCs were treated with IL-1 β (25 ng/mL) for 18 h and the iNOS level was determined by using Western blotting analysis (Figures 1C and 1D). This treatment did not increase the iNOS expression level, suggesting that iNOS, like COX-2, might not be a mediator of the cellular response to IL-1 β in ESMC.

Although there was no change in the COX and iNOS levels, which is similar to other reports, IL-1 β activated p44/42 MAP kinase with increasing concentration (Figure 2). After a treatment with 0.5-50 ng/mL IL-1 β for 3 h, p44/42 MAP kinase was phosphorylated at concentrations above 10 ng/mL. Densitometric assessments demonstrated that the exposure of ESMCs to 25 ng/mL IL-1 β for 3 h significantly increased the level of

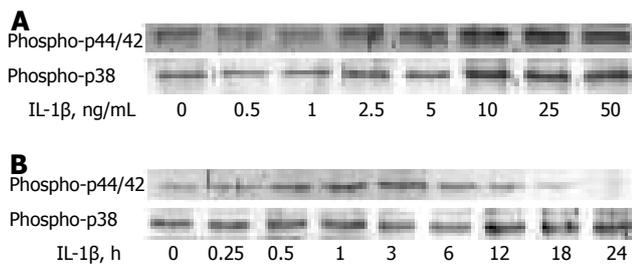


Figure 2 Concentration- and time-dependent activation of MAP kinases after IL-1 β treatment. **(A)** To obtain dose-dependent course of p44/42 MAP kinase and p38 MAP kinase, the cat ESMCs were treated with IL-1 β for 3 and 18 h, respectively. The levels of p44/42 MAP kinase and p38 MAP kinase phosphorylation were increased above a dose of 10 ng/mL, which was maintained up to 50 ng/mL. **(B)** After treatment the cells with IL-1 β (25 ng/mL), the level of p44/42 MAP kinase phosphorylation reached a maximum within 3 h with a subsequent decrease to the basal level (upper panel). The phosphorylated forms of p38 MAP kinase were increased within 12 h, which were sustained to 24 h.

activation of p44/42 MAP kinase by 2.9 ± 0.1 times over the level of control samples (Figure 2A). Furthermore, IL-1 β (25 ng/mL) produced a time-dependent increase in p44/42 MAP kinase phosphorylation (Figure 2B). The effect of IL-1 β on p44/42 MAP kinase was observed within 30 min. The maximum increase (2.6 ± 0.1 -fold increase over the control) in this response was observed within 3 h, and a gradual decrease to the basal level within 18 h was observed. Based on the concentration-response and time-course data, the ESMCs were exposed to IL-1 β at a final concentration of 25 ng/mL for 3 h in later experiments, aiming at identifying the signaling pathway of p44/42 MAP kinase activation. Similarly, p38 MAP kinases were activated by IL-1 β in the cat ESMCs. As shown in Figure 2A, an 18-h IL-1 β treatment caused phosphorylation of p38 MAP kinase in a concentration-dependent manner. A clear increase (1.9 ± 0.1 -fold) was observed at concentrations above 10 ng/mL IL-1 β . The time course of p38 MAP kinase phosphorylation indicated that the IL-1 β increased the activity within 12 h, which was maintained up to 24 h (1.5 ± 0.04 -fold increase over the control). Therefore, even though IL-1 β activated p38 MAP kinase as well as p44/42 MAP kinase, a difference in the activated time of the two MAP kinases was also observed. This suggests that IL-1 β can evoke these cellular responses via at least two pathways, involving p38 MAP kinase and p44/42 MAP kinase, in the cat ESMCs.

In order to determine if the effects of IL-1 β on p44/42 and p38 MAP kinases were mediated through the activation of a receptor coupled to the pertussis toxin (PTX)-sensitive G protein, the ESMCs were pretreated with 100 ng/mL PTX for 24 h, followed by stimulation with IL-1 β for 3 and 18 h, respectively (Figure 3A). Previous studies showed that various types of G proteins, such as Gs, Gq, Go and Gi-3, could be detected in the cat ESMCs^[24]. As shown in Figure 3A, pretreatment of these cells with PTX caused a slight attenuation of p44/42 phosphorylation, indicating that IL-1 β exerts its effects through the receptor-coupled Gi protein. However, p38 MAP kinase phosphorylation was maintained without being mediated by the Gi protein. In order to determine the involvement of adenylate cyclase (AC) in the activation

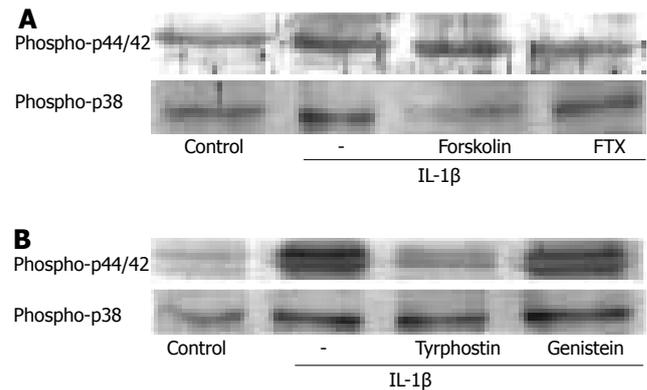


Figure 3 Relation of Gi protein and tyrosine kinase with activation of p44/42 MAP kinase, and negative regulation of p38 MAP kinase by adenylate cyclase. **(A)** Pretreatment with the pertussis toxin (100 ng/mL, 24 h) decreased the density of phosphorylated p44/42 MAP kinase only. On the other hand, forskolin (10 μ mol/L) attenuated p38 MAP kinase phosphorylation by IL-1 β . **(B)** The receptor tyrosine kinase inhibitor, typhostin 51 (30 μ mol/L), markedly reduced the IL-1 β -induced phosphorylation of p44/42 MAP kinase. The activation of p38 MAP kinase was unrelated to the tyrosine kinases.

of MAP kinases, the cells were pretreated with forskolin 24 h before IL-1 β in order to activate the AC (Figure 2A). AC had little effect on p44/42 MAP kinase activation, but was found to decrease the phosphorylated forms of p38 MAP kinase. Therefore, it is believed that IL-1 β -induced p38 MAP kinase activation is negatively regulated by cAMP.

Recently, it has been proposed that IL-1 β causes the activation of tyrosine kinase and regulates protein tyrosine phosphorylation^[22]. There are two classic tyrosine kinases, namely, receptor and nonreceptor tyrosine kinase. The receptor tyrosine kinases are actually the growth factor receptors located in the inner side of the cytoplasmic membrane and undergo dimerization and autophosphorylation upon activation. In this study, we examined the possibility that the observed increase in the level of MAP kinases phosphorylation after an IL-1 β treatment might be mediated by tyrosine kinase (Figure 3B). Therefore, the cells were pretreated with genistein for 1 h prior to adding IL-1 β . This resulted in a decrease in IL-1 β -induced p44/42 MAP kinase activation. In addition, typhostin 51, which is a member of the genistein family of tyrosine kinase inhibitors and is specific to the receptor tyrosine kinase, significantly decreased the level of p44/42 MAP kinase phosphorylation, suggesting that tyrosine kinase might participate in these responses to IL-1 β .

The involvement of phospholipase in the IL-1 β -induced p44/42 MAP kinase activation was examined using a phospholipase C (PLC) inhibitor (neomycin), a PLA₂ inhibitor (DEDA), and a PLD inhibitor (α CMB). As shown in Figure 4A, pretreatment of the ESMCs with neomycin attenuated the p44/42 MAP kinase activation. DEDA also slightly decreased the band density, but could not reach statistical significance. In addition, pretreatment of the ESMCs with α CMB did not decrease the level of p44/42 MAP kinase activation induced by IL-1 β , suggesting that IL-1 β -stimulated p44/42 activation in ESMC might be mediated via the activation of PLC. In this study, we therefore investigated whether or not

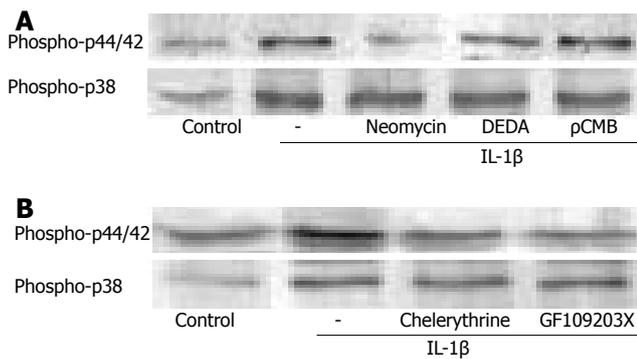


Figure 4 IL-1 β -induced activation of p44/42 MAP kinase is mediated by PLC and PKC. (A) Neomycin (10 μ mol/L, 1 h) decreased the phosphorylated forms of p44/42 MAP kinase. DEDA (10 μ mol/L, 1 h) and pCMB (10 μ mol/L, 1 h) had no effect on the IL-1 β -induced activation of p44/42 MAP kinase. (B) PKC inhibitors chelerythrine (10 μ mol/L, 1 h) and GF109203X (10 μ mol/L, 1 h) decreased the active form of p44/42 MAP kinase. All the phospholipase inhibitors and PKC inhibitors failed to regulate the activation of p38 MAP kinase by IL-1 β .

phosphokinase C (PKC) activation enhanced the p44/42 MAP kinase levels. The ESMCs were pretreated with the PKC inhibitor, GF109203X and chelerythrine (Figure 4B), which reduced the level of p44/42 MAP kinase phosphorylation induced by IL-1 β . It was assumed that there was no relationship between p38 MAP kinase phosphorylation and the phospholipases (Figure 4A). As the pretreatment with the phospholipase inhibitors had no effect on phospho-p38 MAP kinase band density, the PKC inhibitors did not decrease the level of activation by IL-1 β (Figure 4B). Overall, IL-1 β might activate p38 MAP kinase in cat ESMCs via a pathway other than the phospholipase- and PKC-associated pathway.

The relationship between the activation of the two MAP kinases was identified after treating the ESMCs with either 10 μ mol/L PD98059 (a synthetic inhibitor of MEK1/2 activation) or 30 μ mol/L SB202190 (a p38 MAP kinase inhibitor) for 1 h (Figure 5A). PD98059 resulted in inhibition of the IL-1 β -induced p44/42 MAP kinase activation. However, SB202190 had little effect, indicating that p44/42 MAP kinase activation in ESMCs was mediated by the upstream kinase MEK, which was not affected by p38 MAP kinase. SB202190 markedly decreased the level of p38 MAP kinase phosphorylation to almost the basal control level, whereas PD98059 had little effects on this activation. This means that the IL-1 β -induced p38 MAP kinase and p44/42 MAP kinase activation might be accomplished by independent pathways.

Although IL-1 β did not enhance the COX-2 and iNOS expression levels, the participation of COX and NOS in the activation of p44/42 MAP kinase by IL-1 β is still unclear. Therefore, the cells were treated with a COX inhibitor, indomethacin, and a NOS inhibitor, L-NAME, for 1 h before IL-1 β stimulation in order to determine whether COX and NOS play a role in the activation (Figure 5B). The increase in the phosphorylated forms of MAP kinases was maintained even with the pretreatment of inhibitors. This suggested that COX and NOS played no role in IL-1 β -induced MAP kinases activation.

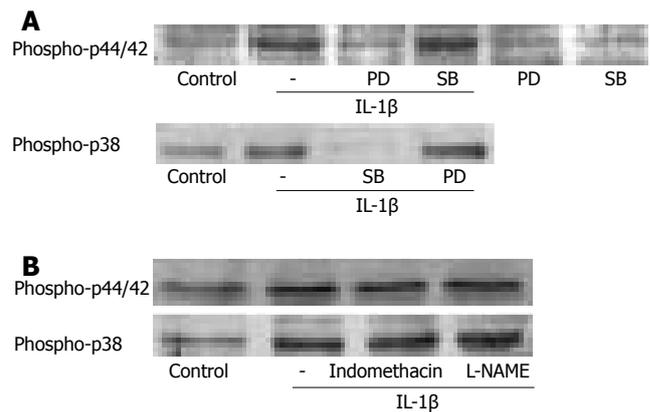


Figure 5 MAP kinase activation by IL-1 β was not affected by other kind of MAP kinase, COX and iNOS. (A) MEK inhibitor PD98059 (10 μ mol/L, 1 h) reduced the level of p44/42 MAP kinase phosphorylation by IL-1 β . SB202190 (30 μ mol/L, 1 h), p38 MAP kinase inhibitor, had no effect on the p44/42 MAPK phosphorylation, which blocked p38 MAP kinase activation. (B) Indomethacin (10 μ mol/L) and L-NAME (10 μ mol/L) had no effect on the activations of p44/42 MAP kinase and p38 MAP kinase by IL-1 β .

DISCUSSION

It is evident that the proinflammatory cytokines and chemokines play an important role in the pathogenesis of various inflammatory conditions^[3,4,25]. Recently, it was reported that there was a significantly higher expression level of IL-8 mRNA in biopsy specimens obtained from RE patients than that from non-inflamed samples, which was determined by competitive reverse transcriptase polymerase chain reaction^[26]. Furthermore, in addition to IL-8, the MCP-1 and RANTES levels were also significantly higher in esophagitis patients. In that report, although there were no significant differences in the mucosal IL-1 β levels between the RE samples and the control group, the tissue IL-1 β levels correlated significantly with the level of IL-8 production^[6]. Cytokines, such as IL-1 β and TNF- α , act locally and systemically to recruit and activate the target cells that can produce additional cytokines. IL-1 induces the gene expression of neutrophils and monocyte chemotactic cytokines, such as IL-8, IL-9, and macrophage inflammatory proteins that in turn stimulate migration and degranulation of neutrophils *in vivo*^[27]. The surgically induced esophagitis rats resulted in an approximately two-fold increase in the level of TNF- α and IL-1 β production^[28].

Previous studies demonstrated that COX-2 and iNOS were the effectors of the IL-1 β -induced cellular responses, products of which were PGE2 and NO, respectively. In our previous study, although iNOS expression and nitrite levels in the tissue were higher after inducing esophagitis in rats (unpublished data), the COX-2 level was unaffected by each IL-1 β concentration ranged from 0.5 to 50 ng/mL. In addition, the IL-1 β treatment by 24 h failed to increase the iNOS concentration in the cultured cat ESMCs. Therefore, IL-1 β did not play a direct role in the inflammatory process in the esophagus, suggesting that IL-1 β might be related to the induction of other cytokines, which would be an immediate cause of the inflammatory cellular response, such as COX-2 and iNOS, in cat ESMC.

IL-1 β plays its own role in the cellular signaling components in the cat ESMC. The MAP kinase pathway has been suggested to be a mechanism by which various signals are transduced from the cell surface to the nucleus in response to a variety of different stimuli. These proteins participate in several intracellular processes by further inducing the level of phosphorylation of various intracellular substrates, such as other protein kinases and transcription factors. This signaling mechanism is believed to control a wide spectrum of cellular physiological and pathophysiological processes including cell growth, differentiation, and the stress response^[29]. The treatment of the ESMC with >10 ng/mL IL-1 β resulted in the activation of p44/42 MAP kinase, which was generated by 30 min and reached a maximum within 3 h, with a subsequent decrease. A similar pattern was observed with p38 phosphorylation, which was also concentration-dependent above 10 ng/mL. This increase was observed after 12 h, suggesting that p44/42 MAP kinase might participate in the early phase response to IL-1 β , and p38 MAP kinase might be a mediator of the late phase IL-1 β effects.

In order to determine whether IL-1 β interacts with the G proteins, the requirement of the G protein was evaluated using ESMC pretreated with the pertussis toxin (PTX). PTX has been shown to inhibit the intrinsic GTPase activity of the G α subunit by the ADP-ribosylation of specific residues. A previous report demonstrated that Gq, G11, Gi1, Gi2, and Gi3 were present in the esophagus and LES^[24]. In this study, the IL-1 β -induced activation of p44/42 MAP kinase was affected by the PTX pretreatment, suggesting the involvement of a PTX-sensitive G protein in these processes. However, PTX had little effect on the IL-1 β -induced activation of p38 MAP kinase. This inhibitory effect of the toxin might result from the increases in the cyclic AMP (cAMP) level via the abrogation of the Gi protein in ESMC. A previous report demonstrated that IL-1 β activated AC in vascular smooth muscle via COX-2^[30]. Therefore, a cAMP elevating agent, forskolin, was used to activate adenylate cyclase in order to evaluate its role in IL-1 β -induced responses. Forskolin failed to attenuate the IL-1 β -induced p44/42 MAP kinase activation, excluding the involvement of elevated cAMP. In contrast, the cAMP concentration affected the level of p38 MAP kinase phosphorylation, suggesting that the delayed activation of p38 MAP kinase is due to the elevation of cAMP by IL-1 β .

It is well known that IL-1 β triggers the activation of PI-PLC and PC-PLC in U937 cells and tracheal smooth muscle cells to generate DAG and IP₃^[31,32]. DAG and IP₃ activate PKC and release Ca²⁺ from the intracellular stores, respectively, and may play an important role in regulating the cellular functions in several cell types^[33]. PKC is a major component in the kinase cascade that is initiated by ligand attachment to both the G protein-coupled receptors and the receptors possessing intrinsic tyrosine kinase activity. Different PKC isozymes appeared to mediate the LES tone as well as the phasic contraction of the ESMC^[34]. In order to confirm the participation of phospholipase in the IL-1 β -induced activation of MAP kinases, the cells were pretreated with inhibitors of each phospholipase.

Neomycin markedly decreased the activity of p44/42 MAP kinase, but failed to reduce the activity of p38 MAP kinase. Therefore, p44/42 MAP kinase and p38 MAP kinase are on a different IL-1 β signaling pathway at least downstream from the receptors. The regulatory mechanisms involved in IL-1 β -induced MAP kinase activation by PKC were further investigated. These results showed that pretreatment with the PKC inhibitors attenuated the IL-1 β -induced p44/42 MAP kinase activation in the ESMC. These results were consistent with those showing that cytokine activated the PLC-coupling signaling pathways in the human histiocytic lymphoma cell line, U937^[31]. Despite the relationship between p44/42 MAP kinase activation by IL-1 β and PKC, the PKC inhibitors did not exert any inhibitory effects on p38 MAP kinase activation.

The implication of a tyrosine kinase in the activation MAP kinases by IL-1 β was investigated using tyrosine kinase inhibitors, genistein and tyrphostin 51. Genistein and tyrphostin 51 inhibited p44/42 MAP kinase phosphorylation, indicating the involvement of tyrosine kinase in this response. In contrast, these inhibitors did not block the phosphorylation of p38 MAP kinase by IL-1 β .

The activation of p44/42 MAP kinase requires both tyrosine and threonine phosphorylation as a result of the dual specificity MEK1/2. PD98059, a synthetic and highly specific MEK1/2 inhibitor, has been shown to inhibit p44/42 MAP kinase activation by several stimuli^[35]. In this study, a PD98059 pretreatment attenuated the p44/42 MAP kinase activation, leaving p38 MAP kinase phosphorylated. In addition, IL-1 β has been shown to phosphorylate p38 MAP kinase, which appears to be distinct from p44/42 MAP kinase in several cell types^[36,37]. SB202190 inhibited the IL-1 β -induced p38 MAP kinase activation, but did not show any inhibitory effect on p44/42 MAP kinase activation. Overall, the activation of p44/42 MAP kinase and p38 MAP kinase appears to involve independent pathways.

The absence of a role of COX and iNOS in these pathways was confirmed by their respective inhibitors, indomethacin and L-NAME. Indomethacin plays a role in preventing COX-2 from producing prostaglandin, and L-NAME blocks NO synthesis by NOS. The IL-1 β -induced activity of p44/42 MAP kinase and p38 MAP kinase remained unchanged even after the indomethacin and L-NAME treatment, thereby excluding the role of prostaglandin and NO in this responses.

In conclusion, IL-1 β , which is believed to act as a pro-inflammatory mediator by evoking other cytokines in the esophagitis, can induce p44/42 MAP kinase and p38 MAP kinase activation. The pathway related to p44/42 MAP kinase is composed of a PTX-sensitive G protein, tyrosine kinase, PLC and PKC. These components are believed to play a role in the early cellular responses via IL-1 β -induced p44/42 activation, but have little effect on p38 MAP kinase phosphorylation, which is generated later and is regulated negatively by cAMP.

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GFAP promoter directs *lacZ* expression specifically in a rat hepatic stellate cell line

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Abstract

AIM: The GFAP was traditionally considered to be a biomarker for neural glia (mainly astrocytes and non-myelinating Schwann cells). Genetically, a 2.2-kb human GFAP promoter has been successfully used to target astrocytes *in vitro* and *in vivo*. More recently, GFAP was also established as one of the several markers for identifying hepatic stellate cells (HSC). In this project, possible application of the same 2.2-kb human GFAP promoter for targeting HSC was investigated.

METHODS: The GFAP-*lacZ* transgene was transfected into various cell lines (HSC, hepatocyte, and other non-HSC cell types). The transgene expression specificity was determined by X-gal staining of the β -galactosidase activity. And the responsiveness of the transgene was tested with a typical pro-fibrotic cytokine TGF- β 1. The expression of endogenous GFAP gene was assessed by real-time RT-PCR, providing a reference for the transgene expression.

RESULTS: The results demonstrated for the first time that the 2.2 kb hGFAP promoter was not only capable of directing HSC-specific expression, but also responding to a known pro-fibrogenic cytokine TGF- β 1 by upregulation in a dose- and time-dependent manner, similar to the endogenous GFAP.

CONCLUSION: In conclusion, these findings suggested novel utilities for using the GFAP promoter to specifically manipulate HSC for therapeutic purpose.

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Key words: GFAP; Astrocytes; Hepatic stellate cells; Fibrosis; TGF- β 1; *LacZ*.

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INTRODUCTION

Hepatic stellate cell (HSC, also known as Ito cell) is a minor cell type (roughly 5-8% of the total liver cells) most commonly found in the sinusoidal area of adult livers. The major physiological functions of HSC include fat storage, vitamin A uptake and metabolism, and the production of extracellular matrix (ECM) proteins. It has been demonstrated in the past decade that HSC is a major player in mounting defense during hepatic injury, and mediating hepatic fibrogenesis by over-producing pro-fibrotic cytokines and consequently the ECM molecules. Hence the HSC itself also became a target for the development of anti-fibrotic therapy^[1-3].

Traditionally antibodies against desmin^[4], vimentin and smooth muscle- α -actin (SMAA)^[5,6], despite their poor tissue and cell specificity, remained the common battery for identifying rat HSC both *in vitro* and *in vivo*, until the re-introduction of GFAP in the mid-1990s^[7-9], even though GFAP was first documented as a marker for rat HSC in 1985^[10]. It was suggested that the GFAP could be a more reliable marker for marking quiescent HSCs^[7]. More recently, the GFAP was also adopted as a marker for human HSCs^[11-13].

Several gene promoters have been investigated for their ability to genetically manipulate HSC both *in vitro* and *in vivo*, which include the human collagen alpha 1 (Coll)^[14-16], SMAA^[17], LIM domain protein CRP2 (CSRP2), tissue inhibitor of metalloproteinases-1 (TIMP-1) and smooth muscle-specific 22-ku protein (SM22 alpha)^[18,19]. However, none of the promoters tested thus far showed sufficient tissue and cell specificity.

In our own efforts, we constructed a transgene consisting of a 2.2-kb hGFAP promoter^[20,21] and the *E. coli lacZ* coding sequence and tested its expression in a rat HSC line HSC-T6^[22]. Our results showed that the 2.2-kb hGFAP promoter was not only capable of directing HSC-specific expression *in vitro*, but also responding to a known pro-fibrogenic cytokine transforming growth factor (TGF- β 1) by upregulation in a dose- and time-dependent

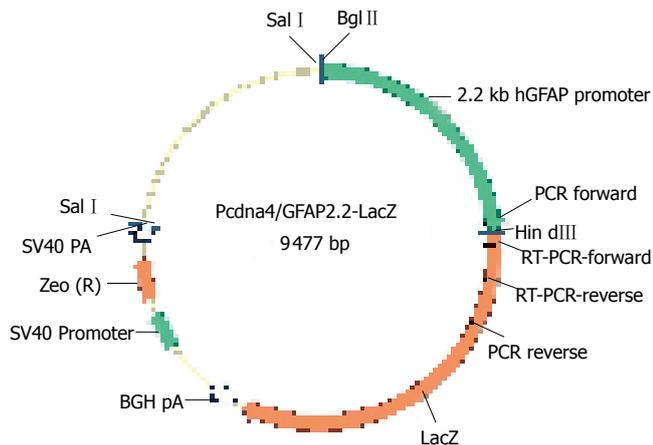


Figure 1 GFAP-*lacZ* transgene construct. The bacterial *lacZ* reporter coding sequence was under the control of a 2.2-kb human GFAP promoter. The SV40-Zeocin gene provides a resistance to antibiotic selection for stable clones. Primer locations for PCR and RT-PCR were also indicated.

manner. Similarly, an induction of the endogenous GFAP by TGF- β 1 was also observed. These findings suggested novel utilities for the 2.2 kb hGFAP promoter for specifically manipulating HSC, and developing anti-fibrotic therapy. Furthermore, these findings raised fundamental questions on possible GFAP function in the HSC development and pathobiology.

MATERIALS AND METHODS

Construction of the 2.2 kb hGFAP-*lacZ* transgene

The plasmid vector pcDNA4/TO/*LacZ* (Invitrogen, CA, USA) was used as a cloning backbone, providing the *E. coli* β -galactosidase coding sequence and the Zeocin resistance gene. The 2.2-kb hGFAP promoter, which was originally mapped for its astrocytic specificity^[20,21], was excised from another transgene GFAP-GFP-S65T^[23] by *Bgl*II/*Hind*III digest and used to replace the CMVtetO2 promoter in the pcDNA4/TO/*lacZ* vector. The cloning junction sequences were verified by DNA sequencing. The resultant 9.5 kb plasmid (as depicted in Figure 1) was designated as pcDNA4/GFAP2.2-*LacZ* in our vector depository.

Cell line and culture conditions

The rat HSC-T6 cell line^[22] was a generous gift from Dr Scott Friedman of the Mount Sinai School of Medicine in New York through Dr Alex Hui (Chinese University of Hong Kong). The HepG2, C3A, HeLa and NIH/3T3 were from American Type Culture Collection (ATCC, VA, USA), and the C6 was from Japanese Collection of Research Bioresources (JCRB, Osaka, Japan). All the cell culture media and reagents were from Invitrogen, unless specified otherwise. All cell lines were routinely cultured in full Dulbecco's modified Eagle medium (DMEM), supplemented with 10% FBS, 100 units penicillin/100 μ g streptomycin per mL at 37°C in a humidified atmosphere of 50 mL/L CO₂. The cells were routinely split twice weekly in a ratio of 1:3.

Cell transfection and selection

The T6, C6, and HeLa cells were stably transfected with

0.5-1.0 μ g of the SalI-linearized transgene plasmid using the Lipofectamine 2000 kit, according to the manufacturer's instructions (Invitrogen). Twenty-four hours after the transfection, the medium was changed and supplemented with 250-500 μ g/mL Zeocin for selection for at least 6 wk before further analyses (as described below) were performed. The final concentration for maintaining the stable transfected cells was 500 μ g/mL Zeocin. Alternatively, HepG2, C3A, and NIH/3T3 were transiently transfected with the circular transgene plasmid using the FuGene 6 kit (Roche Diagnostics).

Genomic detection of the transgene

In order to confirm the transgene integrity and its integration into the genome, genomic DNA was isolated from cell clones stable for at least 2 mo under 500 μ g/mL Zeocin selection, using the NucleoSpin blood kit (Macherey-Nagel, Düren, Germany). A PCR method was used to verify the structural integrity of the inserted construct(s), with a forward primer 5'-ACTCCTTCATAAAGCCCTCG-3' (complementary to the GFAP promoter), and a reverse primer 5'-AACTCGCCGCACATCTGAACTTCAGC-3' (complementary to the *lacZ* coding sequence), using the Platinum PCR SuperMix High Fidelity (Invitrogen) on a thermal cycler (MJ Research, FL, USA). The expected PCR product was 944 bp in size.

RT-PCR GFAP-*lacZ* transcript

Total cellular RNAs were extracted from cells grown in 6-well plates by using the NucleoSpin RNAII kit (Macherey-Nagel) following the manufacturer's instructions. The RNA concentration was determined on a ND-100 spectrophotometer (Nanodrop Technologies, DE, USA). Fifty nanograms of total RNA was used to perform a one-step RT-PCR (Qiagen, Hilden, Germany) according to the user's manual, using forward primer (5'-TCAGCTTG GAGTTGATCCCGTTCG-3') and reverse primer (5'-AAC AACGGCGGATTGACCGTAATGG-3'). The thermal cycling profiles were 50°C 30 min (cDNA synthesis), 95°C 15 min (denaturation), and followed by 94°C 10 s 55°C 10 s, and 72°C 19 s for 35 cycles (PCR). The target product size was 337 bp, and the β -actin reference was 332 bp in size.

X-gal staining of β -galactosidase activity in fixed cells

A *lacZ* reporter cell staining kit (InvivoGen, CA, USA) was used to detect the reporter gene activity in both stable and transient transfectants. Development of the blue end product was monitored at different time intervals (15, 30, 60, 90, and 120 min). The staining results were documented with a digital camera (DP12) attached to an Olympus inverted bright field microscope (IX51).

Quantitative solution assay of β -galactosidase activity in cell extracts

To quantify the β -galactosidase activity in cell extracts, an enzyme assay kit (E2000, Promega, WI, USA) was used to measure the specific activity of β -galactosidase, according to the manufacturer's instructions in a 96-well format. Briefly, the cells were washed twice with 1 \times PBS buffer (pH 7.4), lysed for 15 min at room temperature with the reporter lysis buffer, and harvested using a cell scraper. The total cell extracts were appropriately diluted

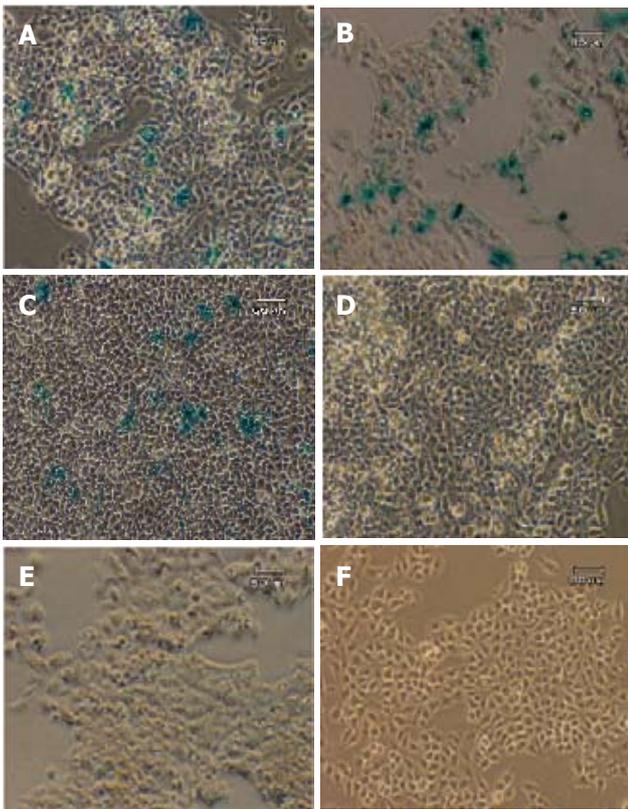


Figure 2 Transient transfection with CMV-*lacZ* and GFAP-*lacZ* transgenes. Hepatocyte cell lines C3A (A), HepG2 (B), and fibroblast cell line NIH/3T3 (C) transfected with the ubiquitous CMV-*lacZ* transgene displayed blue color after 2 h of X-gal staining. In contrast, C3A (D), HepG2 (E) and NIH/3T3 (F) transfected with the GFAP-*lacZ* transgene did not show any blue color. Scale bar=60 μ m.

(5-10 folds), and assayed for the enzyme activity. The specific β -galactosidase activity was presented in milliunit per milliliter (mU/mL) using a standard curve established from the β -galactosidase standard (provided with the kit). The protein concentration was measured with a BCA protein assay kit (Pierce, IL, USA), and used to further convert the specific β -galactosidase activity from mU/mL to mU/ μ g total cellular protein. Final data were summed from six independent experiments.

Treatment of cells with TGF- β 1

Cells were seeded into 12-well plates in full DMEM with 500 μ g/mL Zeocin. Twelve hours prior to the cytokine treatment, medium was changed so that the cells were allowed to grow in low serum (0.5% FBS) DMEM. The cells at a confluence of 70-80% were incubated with various concentrations (0, 0.1, 1, and 10 ng/mL) of rhTGF- β 1 (BioVision, CA, USA) for 0, 2, 8, 16, 24, 48, and 72 h before they were harvested for β -galactosidase activity assay and real-time RT-PCR GFAP assay.

Real-time RT-PCR quantification of rat GFAP mRNA

Total RNA was reversely transcribed to cDNA using Taqman's reverse transcription reagent (Cat. # N808-0234). A total of 400 ng of RNA in 7.7 μ L nuclease-free water was added to 2 μ L 10 \times reverse transcriptase buffer, 4.4 μ L 25 mmol/L magnesium chloride, 4 μ L deoxyNTP mixtures, 1 μ L random hexamers, 0.4 μ L RNase inhibitor

and 0.5 μ L reverse transcriptase (50 U/ μ L) in a final reaction volume of 20 μ L. The reaction was performed for 10 min at 25 $^{\circ}$ C (annealing), 30 min at 48 $^{\circ}$ C (cDNA synthesis) and 5 min at 95 $^{\circ}$ C (enzyme denaturation).

Real-time quantitative PCR was carried out with an ABI 7500 Real Time PCR System (Applied Biosystems, CA, USA). One microliter of sample cDNA was used in each PCR reaction, with the actin gene as a reference. The primers and probes for rat β -actin and GFAP were purchased from Taqman's assay-on-demand database. The PCR reaction was performed under a default profile consisting of 50 $^{\circ}$ C for 2 min (UNG activation), 95 $^{\circ}$ C for 10 min (enzyme denaturation) and 40 cycles of amplification (denaturation 15 s, annealing and extension 60 s).

Relative quantitation of the target mRNA was calculated using the comparative threshold cycle (C_T) methods as described in the User Bulletin #2 (ABI Prism 7700 Sequence Detection System). C_T indicates the fractional cycle number at which the amount of amplified target reaches a fixed threshold within the linear phase of gene amplification. ΔC_T , which reflects the difference between C_T target and C_T β -actin, is inversely correlated to the abundance of mRNA transcripts in the samples. ΔC_T for each sample was normalized against control experiment or calibrator and expressed as $\Delta\Delta C_T$. Relative quantitation is given by $2^{-\Delta\Delta C_T}$ to express the upregulation or downregulation of the target gene under the treatments compared to the control.

Six independent experiments were performed for each data point and three ΔC_T were measured for each experiment.

Statistical analysis

All quantitative results were presented as mean \pm SE. Experimental data were analyzed using two-tailed Student's *t*-test assuming unequal variances. A *P*-value < 0.05 was considered significant.

RESULTS

Lack of GFAP-*lacZ* expression in non-GFAP-expressing cell lines

To investigate if there is any possible aberrant expression of the transgene, three non-GFAP expressing cell lines, HepG2, C3A and NIH/3T3, were transiently transfected with the circular transgene plasmid, using a CMV-*lacZ* plasmid as a positive control. After the transfected cells were grown in full DMEM medium free of selection agent for 2 days, all three cell lines were examined for the β -galactosidase expression by X-gal staining method. Approximately 10-30% of the cells in each of the three lines transfected with the ubiquitous CMV-*lacZ* showed positive blue color after 2 h of staining. In contrast, none of the three lines transfected with the GFAP-*lacZ* showed any blue staining, indicating a total lack of *lacZ* expression driven by the GFAP promoter in these cell lines. Representative images from two independent experiments for all three cell lines are shown in Figure 2. After 24 h of X-gal staining, a few cells from the HepG2 line displayed blue color in both the transfected and

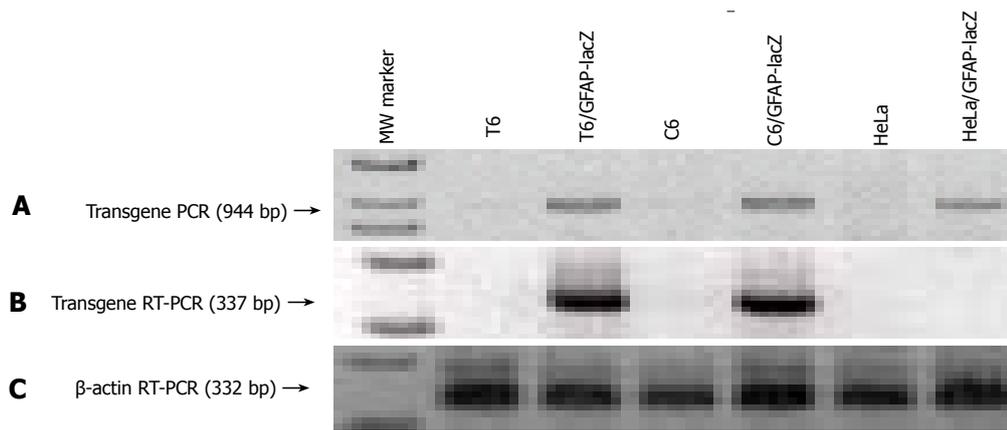


Figure 3 Verification of GFAP-*lacZ* transgene integration into host genome and legitimate expression in appropriate cell types. **A:** PCR of genomic DNA isolated from the transfected T6 (lane 3), C6 (lane 5) and HeLa (lane 7) showed the presence of a transgene band at 944 bp, and the absence of such band from the nontransfected T6 (lane 2), C6 (lane 4), and HeLa (lane 6); **B:** RT-PCR of total RNAs demonstrated that transgene only expressed in the transfected T6 (lane 3) and C6 (lane 5), but not in the transfected HeLa (lane 7); **C:** RT-PCR of β -actin as a reference ensuring equal sample loading. Lane 1 is the 1-kb ladder marker.

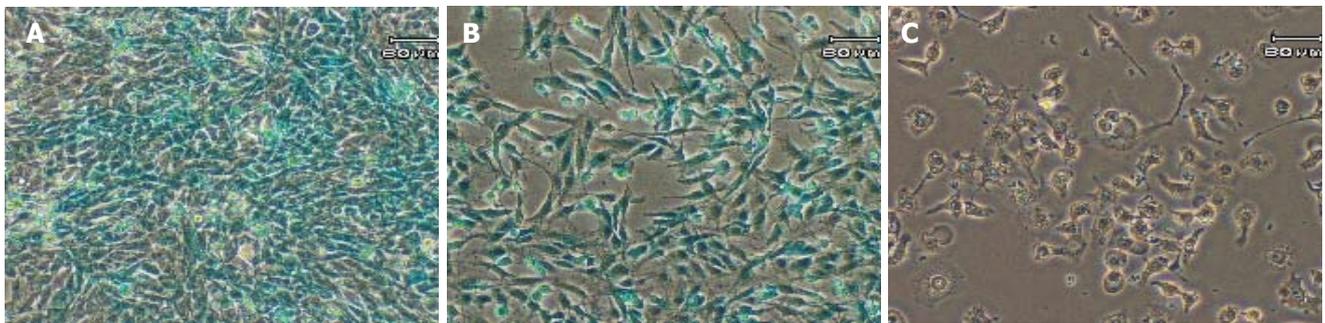


Figure 4 X-gal staining of stable transfectants. The GFAP-*lacZ* transgene expressed in the rat hepatic stellate cell line T6 (A), and the positive control rat astrocytic cell line C6 (B), but not in the non-GFAP-expressing cell line HeLa (C). Scale bar = 60 μ m.

nontransfected groups alike (data not shown), most likely due to endogenous galactosidase-like activity.

Specific expression of GFAP-*lacZ* in GFAP-expressing cell line T6

The 2.2-kb hGFAP promoter has been consistently shown to direct gene expression specifically in the neural stellate cells-astrocytes^[20,21,24]. It was completely unknown whether a GFAP promoter can direct gene expression specifically in the HSC type. Since stellate cells of both neural and hepatic origins have been reported to share many morphological, immunocytochemical and even developmental features^[7-9,25], we decided to test this possibility by stably transfecting a rat HSC line T6^[22] with the GFAP-*lacZ* transgene, using the rat astrocytic C6 cell line as a positive and the human HeLa cell line as a negative control. After selection in 250-500 mg/mL Zeocin for at least 6 wk, several independent stable cell clones were obtained for each cell line. Subsequently, PCR analysis of genomic DNA isolated from the stable clones confirmed the transgene integrity and integration into the host genome, as evidenced by the presence of an anticipated product size of 944 bp (Figure 3A). To check for transgene expression, total RNA was isolated from the stable transfectants and RT-PCR was performed to

verify for the *lacZ* transcript. The presence of a specific band with a predicted size of 337 bp indicated the *lacZ* transcription in the positive control C6 line, and more importantly in our target T6 line as well, but not in the negative control line HeLa (Figure 3B). The β -actin sample is shown in Figure 3C as a control for equal RNA loading. Next, one clone from each of the three cell lines was randomly chosen for X-gal staining for 1 h. As expected, both the C6 and the T6 were positive and the HeLa was negative in blue staining (Figure 4). Therefore for the first time, a GFAP-based reporter gene was shown to specifically express in a HSC line. The representative staining results are shown in Figure 4. From this point onward, a T6 cell clone stably transfected with the 2.2 kb hGFAP-*lacZ* transgene (designated as T6/*lacZ*/C1 clone) was used for further experiments below. It was worth noting that the T6 cells transiently transfected with the GFAP-*lacZ* transgene stained positive for X-gal reaction as well.

Time- and dose-dependent induction of transgene by TGF- β 1 in T6/*lacZ*/C1 cells

TGF- β 1 has been reported to exert broad biological effects on the cells, including the regulation of GFAP in astrocytes^[26-29], and the fibrogenesis in the

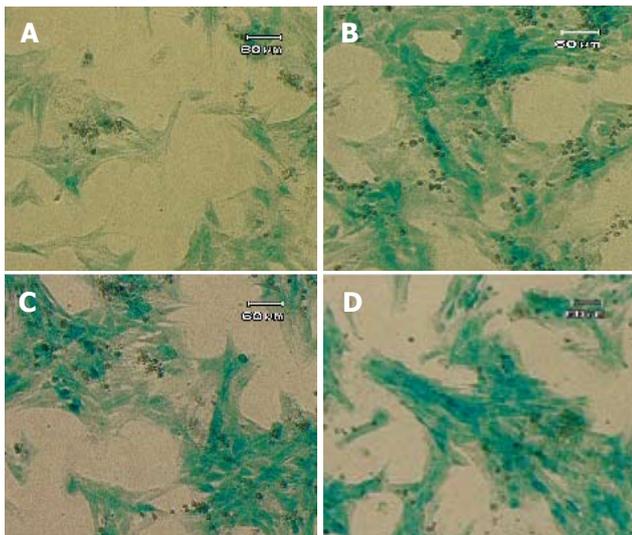


Figure 5 Induction of GFAP-*lacZ* transgene expression by TGF- β 1. The T6/*lacZ*/C1 cells were cultured and incubated in full DMEM supplemented with 0 (A), 0.1 (B), 1 (C), and 10 (D) ng/mL of TGF- β 1 for 3 days, and stained with X-gal substrate for 1 h. Cells treated TGF- β 1 displayed more intense blue staining and more activated morphology (thicken processes). Scale bar = 60 μ m.

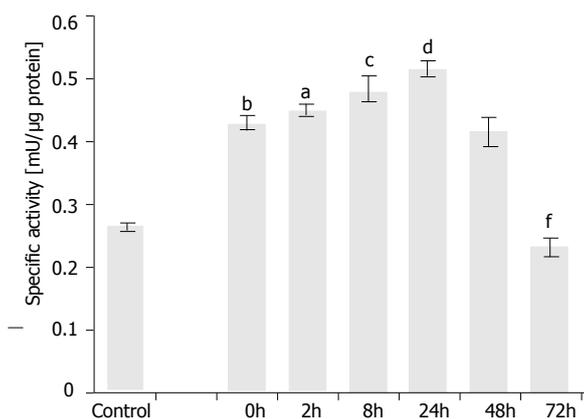


Figure 6 Time-dependent induction of GFAP-*lacZ* gene by TGF- β 1 in the T6/*lacZ*/C1 cells. Cells were grown in full DMEM was assayed for basal β -galactosidase activity (control). Cells adopted in low serum DMEM for 12 h were supplemented with 1 ng/mL of TGF- β 1 for various times (0, 2, 8, 24, 48, and 72 h), and assayed for enzymatic activity. When compared to the basal level, cells cultured in low serum DMEM had elevated level of transgene expression ($^bP < 0.001$). When compared to the 0 h, transgene expression level was significantly induced at 2 h ($^aP < 0.05$), 8 h ($^cP < 0.005$), and 24 h ($^dP < 0.001$), but significantly reduced at 72 h ($^fP < 0.001$) compared with 0 h. Data presented as mean \pm SE.

HSC^[30-33]. However, very little was known about the potential regulation of GFAP (and GFAP-based transgene) by the potent pro-fibrogenic TGF- β 1 in HSC. To obtain a first hint, the T6/*lacZ*/C1 cells were treated with TGF- β 1 at various concentrations (0, 0.1, 1, and 10 ng/mL) in the full DMEM medium for 72 h. The cells were then stained with X-gal for one hour and photographed. When compared to the untreated cells, cells cultured with TGF- β 1 displayed a more intense blue staining and activated morphology (thicken cellular processes), as shown in Figure 5.

To examine time-dependent induction, the T6/*lacZ*/C1 cells stimulated with 1 ng/mL of TGF- β 1 for

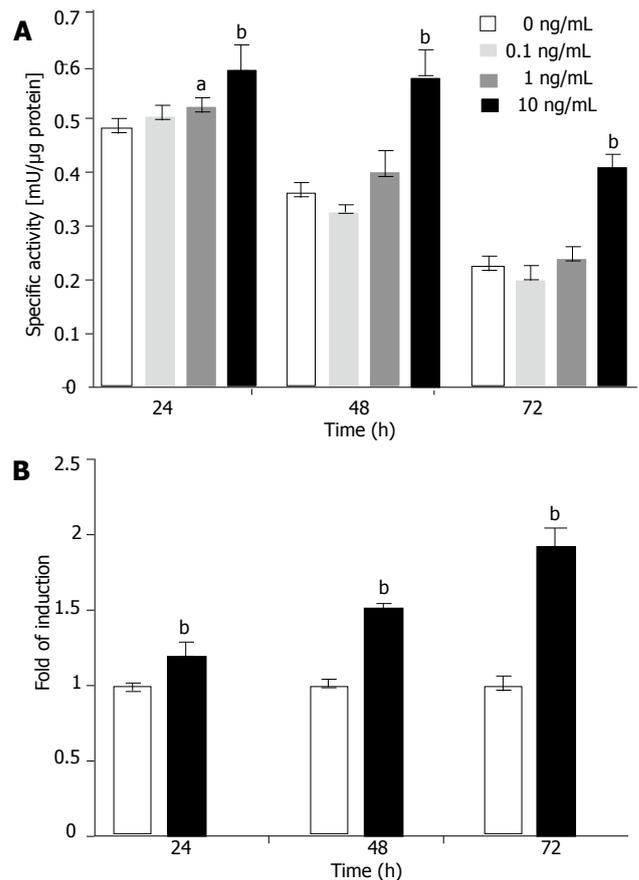


Figure 7 Dose-dependent induction of transgene. (A) The T6/*lacZ*/C1 cells cultured in low serum DMEM supplemented with different concentrations of TGF- β 1 (0, 0.1, 1, and 10 ng/mL) for various times (24, 48, and 72 h) were assayed for β -galactosidase activity. The transgene was significantly induced by 10 ng/mL of TGF- β 1 at all time points. (B) Data extracted from (A) illustrated that the fold of induction increased with times, despite the drop in absolute enzymatic activity. Data presented as mean \pm SE. $^aP < 0.05$, $^bP < 0.001$ compared with no cytokine treatment.

various times (0, 2, 8, 24, 48, and 72 h) were assayed for β -galactosidase activity. Firstly, it was interesting to note that the transgene expression was significantly increased ($P < 0.001$) from 0.29 mU/ μ g to 0.42 mU/ μ g (or a 24% increase) by simply lowering the serum concentration from 10% to 0.5% and culturing for 12 h. This acute and transient increase in the transgene expression could be a stress response for cells adopted from full- to lower-serum medium. Secondly, under 1 ng/mL of TGF- β 1 the transgene was rapidly induced when measured at 2 h ($P < 0.05$) and 8 h ($P < 0.005$), and eventually peaked at 24 h ($P < 0.001$), with a specific activity being 0.52 mU/ μ g. At 48 h, the expression level dropped to the same level as at 0 h. After 72 h, the level slipped to 58% of the value at 0 h ($P < 0.001$). The assay results are depicted in Figure 6.

To investigate dose-dependent induction, the T6/*lacZ*/C1 cells were grown in low serum DMEM with various concentrations of TGF- β 1 (0, 0.1, 1, and 10 ng/mL) for various times (24, 48, and 72 h) and assayed for β -galactosidase activity. Significant transgene induction was observed for the 10 ng/mL TGF- β 1 treatment at all time points ($P < 0.001$). However the only other (less) induction was seen with 1 ng/mL of TGF- β 1 at 24 h ($P < 0.05$). The assay results are plotted in Figure 7A. It is

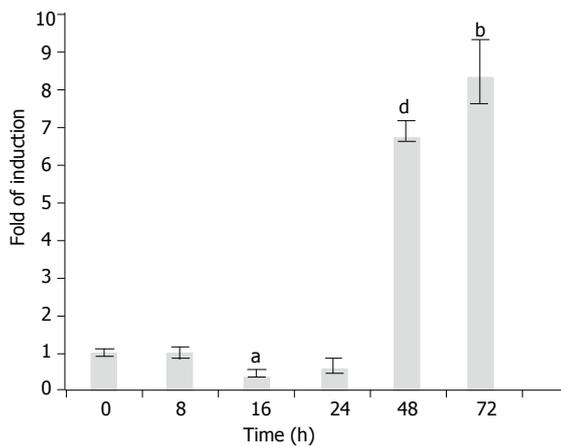


Figure 8 Time-dependent induction of the endogenous GFAP gene by TGF- β 1. T6/GFAP-LacZ cells were incubated with 1 ng/mL of TGF- β 1 in low serum DMEM for various times (0, 8, 16, 24, 48, and 72 h), and the endogenous rat GFAP mRNA was quantified with real-time RT-PCR. Data were presented as mean \pm SE. ^a $P < 0.01$, ^b $P < 0.005$ and ^d $P < 0.001$, when compared to treatment at 0 h.

interesting to note that when the values of control groups (for 10 ng/mL) were normalized to 1 and the highest induction (nearly two fold) was seen at 72 h, though the absolute enzymatic activity (0.43 mU/ μ g) was the lowest among the three time points (Figure 7B).

Induction of endogenous GFAP expression by TGF- β 1

The GFAP, along with other HSC markers desmin, SMAA, and vimentin, was known to express in the HSC-T6 cells^[22]. In order to investigate whether the endogenous GFAP expression is also subject to TGF- β 1 regulation as the transgene, the T6/lacZ/C1 cells treated with TGF- β 1 (1 ng/mL) for various times (0, 2, 8, 16, 24, 48, and 72 h), and the rat GFAP transcripts were quantified using a real-time RT-PCR method. When compared to the basal level (normalized to 1) at 0 h, the GFAP mRNA level remained relatively steady within the first 24 h (except a brief but significant 0.5-fold suppression at 16 h, $P < 0.005$), then sharply elevated to 7- and 8.5-folds of the basal level at 48 and 72 h, respectively (Figure 8).

DISCUSSION

Cell specificity of transgene expression

To our best knowledge, this is the first report demonstrating that a GFAP promoter can drive HSC-specific expression of a reporter gene, in a similar fashion as the endogenous GFAP does. The current findings also provided molecular biology support to the early immunocytochemical observations on the GFAP staining on HSC^[7-11,36,37]. Based on our transfection studies of multiple cell types, the 2.2 kb hGFAP promoter is both necessary and sufficient to confer HSC-specific expression *in vitro*, which was reminiscent to the conclusion made on cultured astrocytes using the same promoter^[20]. Several key *cis*-acting elements, especially the AP-1 site (for binding fos/jun family transcription factors), have been mapped to the so-called B box in the human GFAP promoter

from position-1612 to-1489, and proved to be critical for mediating astrocyte-specific transgene expression^[20,38,39]. We therefore attempted to speculate that the rat T6 cells should also express a similar set of relevant transcription factors as the astrocytes do, to permit selective GFAP-lacZ expression in HSC. On the contrary, the non-GFAP expressing cell lines (HepG2, C3A, HeLa, and NIH/3T3) may lack appropriate *trans*-acting factor profile, leading to the complete null expression of the transgene, as judged by RT-PCR and prolonged X-gal staining.

GFAP promoter elements for mediating the induction by TGF- β 1

Our quantitative data showed that the 2.2 kb hGFAP-lacZ transgene, as well as the endogenous rat GFAP, were induced by TGF- β 1 in a time- and dose-dependent manner in the T6/lacZ/C1 cells. This observation on the HSC was similar to earlier reports on the astrocytes^[28,40]. The molecular mechanism underlying the TGF- β 1 induction has been extensively studied. A sequence motif resembling the NF-1 (nuclear factor-1) site located in the near upstream (-106 to -153) of the rat GFAP promoter was shown to confer a full response to TGF- β 1 induction in cultured astrocytes^[40]. Similarly, two NF-1-like sites were mapped to the B (-1612 to -1489) and D (-132 to -57) boxes in the human GFAP promoter^[20]. It was also known that some factor(s) in the nuclear extracts from astrocytes could bind to the NF-1 sites and possibly mediates the TGF- β 1 induction process.

The endogenous GFAP gene displayed a similar trend in response to TGF- β 1 as the 2.2 kb GFAP-lacZ transgene did. However, different responding dynamics were noted between the endogenous GFAP gene and the artificial GFAP-lacZ transgene. First, the transgene induction was obvious within the first 2 h of TGF- β 1 stimulation, while the endogenous gene did not show any significant induction until day 2 of the TGF- β 1 treatment. The difference in induction time line could be partially due to assay detection sensitivities, presumably with the enzymatic assay (with amplification capability) being more sensitive, therefore showing an earlier induction. An earlier detection of a similar 2.2-kb hGFAP-lacZ transgene was documented in a transgenic mouse model during brain development, in which the transgene showed up several days ahead of the endogenous GFAP^[21]. In addition, unknown regulatory elements residing outside the 2.2-kb fragment could also contribute to the different responding profiles between the transgene and the endogenous GFAP. Nevertheless, both the endogenous and transgene at least displayed the same upward trend in responding to the same TGF- β 1 cytokine, legitimizing the use of GFAP-lacZ transgene as a surrogate for the endogenous GFAP in studying TGF- β 1 stimulation.

Relationship between HSC activation and GFAP (and GFAP-lacZ) expression

Early studies on the rat HSC showed that GFAP expression decreased as quiescent HSCs were activated or transdifferentiated to myofibroblast-like during fibrosis^[7-9]. However, the notion of an inverse relationship between the GFAP expression and the HSC activation may not be

as simple and straightforward. For example, whether the activated HSCs and myofibroblasts *in vivo* represented different activation stages of the same cell lineage, or two distinct cell types of different origins remained controversial^[41]. It is worthy noting that some recent data showed that activated HSCs expressed higher level of GFAP^[11,34,37]. Our current data clearly showed that both the endogenous GFAP and the hGFAP-*lacZ* transgene were upregulated in a time- and dose-dependent manner in the rat HSC-T6 cells activated by TGF- β 1.

Our data demonstrated for the first time that the 2.2 kb hGFAP promoter is capable of expressing the *lacZ* reporter specifically in the rat HSC-T6 line and responding to TGF- β 1 activation by re-regulation, in a similar manner as the rat astrocytic cell line C6. Furthermore our data also suggested that the molecular and cellular mechanisms (relevant *trans*-acting factors and signaling pathways) underlying the specific GFAP expression and induction are likely conserved in HSC line T6 and in the neural stellate cell line C6 alike. The current findings should provide a valuable genetic tool for manipulating HSCs towards the development of anti-fibrotic therapies.

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Variable expression of cystatin C in cultured trans-differentiating rat hepatic stellate cells

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Abstract

AIM: To study the expression of cystatin C (CysC), its regulation by transforming growth factor- β 1 (TGF- β 1) and platelet-derived growth factor (PDGF) and the potential interference of CysC with TGF- β 1 signaling in this special cell type.

METHODS: We evaluated the CysC expression in cultured, profibrogenic hepatic stellate cells and trans-differentiated myofibroblasts by Northern and Western blotting and confocal laser scanning microscopy.

RESULTS: CysC was increased significantly in the course of trans-differentiation. Both TGF- β 1 and PDGF-BB suppressed CysC expression. Furthermore, CysC secretion was induced by the treatment with TGF- β 1. Although CysC induced an increased binding affinity of TGF- β receptor type III (beta-glycan) as assessed by chemical cross-linking with [125 I]-TGF- β 1, it did not modulate TGF- β 1 signal transduction as shown by evaluating the Smad2/3 phosphorylation status and [CAGA]-MLP-luciferase reporter gene assay. Interestingly, the shedding of type III TGF- β receptor beta-glycan was reduced in CysC-treated cells. Our data indicated that CysC expression was upregulated during trans-differentiation.

CONCLUSION: Increased CysC levels in the serum of patients suffering from liver diseases are at least partially due to a higher expression in activated hepatic stellate cells. Furthermore, TGF- β 1 influences the secretion of CysC, highlighting a potentially important role of cysteine proteases in the progression of hepatic fibrogenesis.

INTRODUCTION

Cystatin C (CysC), belonging to the type II cystatin gene superfamily, is the most abundant extracellular inhibitor of cysteine proteinases^[1]. Mature CysC is a 13-kD, positively charged, secreted protein composed of 120-122 amino acids expressed by many cell types. It is present in large quantities in cerebrospinal fluid, seminal plasma, serum and other body fluids. One of the most prominent functions of CysC is related to the inactivation of the cathepsin family members of cysteine proteinases, which are involved in the cleavage of membrane and extracellular matrix proteins among others and thus in disease-related tissue remodeling. The diagnostic value and prognostic significance of CysC determination have been reported for several diseases^[2,3], and correlations have been found between CysC expression, mutations, and clinical status of patients with autoimmune disease, cerebral amyloid angiopathy, hereditary brain hemorrhage, atherosclerosis, aortic aneurysms, and multiple sclerosis^[4-8].

A further highly relevant function of CysC has been recently reported, which is concentrated on the inhibitory effect on transforming growth factor- β (TGF- β)-signal transduction in normal and malignant cells^[9]. The TGF- β antagonizing mechanism is at least partially due to the interference of ligand binding to the type II TGF- β receptor. *In vitro*, TGF- β 1 is a potent inducer of CysC secretion in vascular smooth muscle cells^[8]. Furthermore, expression of CysC mRNA in astrocyte precursor cells is directly linked to the activity of TGF- β ^[10]. TGF- β is known to be the fibrogenic master cytokine in human and experimental liver fibrosis due to its ability to stimulate the expression and inhibition of degradation of extracellular matrix (ECM) proteins and to promote the trans-differentiation of profibrogenic hepatic stellate cells (HSCs) to ECM-producing hepatic myofibroblasts (MFBs)^[11]. The phenotypic and functional trans-differentiation of

HSCs is initiated in acute and chronic inflammatory liver diseases, ultimately leading to organ fibrosis and cirrhosis. Significantly elevated serum concentrations of CysC have been recently described in chronic liver diseases showing a strong correlation between the degree of elevation and the severity of disease^[12,13]. Based on these new findings, we tried to figure out the potential functional relevance of CysC in the profibrogenic liver cell subtype, HSCs. Therefore, we studied in cultured rat HSCs, the expression of CysC, its regulation by TGF- β 1 and platelet-derived growth factor (PDGF) and the potential interference of CysC with TGF- β signaling in this special cell type. The results showed that CysC expression in HSCs was modulated by TGF- β and platelet-derived growth factor-BB (PDGF-BB) depending on the trans-differentiation status of the cells. However, CysC had no direct effect on TGF- β signaling itself.

MATERIALS AND METHODS

Cell isolation and culture

HSCs, Kupffer cells (KCs), and sinusoidal endothelial cells (SECs) were isolated from male Sprague-Dawley rats by the pronase-collagenase method^[14]. HSCs were further purified by a single-step density gradient centrifugation as previously described^[15]. KCs and SECs were enriched by centrifugal elutriation^[16] and RNA was directly isolated without cell cultivation. HSCs were seeded in Dulbecco's modified Eagle's medium (DMEM) (BioWhittaker Europe, Verviers, Belgium) supplemented with 100 mL/L fetal calf serum (FCS) (Hyclone Fetal Bovine Serum, Perbio), 4 mL glutamine (PAA Laboratories GmbH, Linz, Austria), 100 IU/mL penicillin and 100 μ g/mL streptomycin (PAA Laboratories). Hepatocytes (PCs) were isolated following the collagenase method of Seglen^[17]. The medium was renewed one day after initial plating and then every day. Cultures were maintained at 37 °C in a humidified atmosphere containing 50 mL/L CO₂. Prior to treatment with recombinant human CysC (rhCysC), TGF- β 1, and PDGF-BB (R&D Systems, Wiesbaden, Germany), the cells were starved for 16 h in 0.2% FCS.

RNA isolation and Northern blot analysis

Total RNA was purified using the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. After resuspension in water, the concentration was determined by UV absorbance. Northern blot analysis was carried out as previously described^[18].

cDNA preparation, RT-PCR, real time PCR

To generate a probe for Northern blot analysis of CysC expression, total RNA (2 μ g) from rat HSCs was reverse transcribed using the Omniscript transcriptase (Qiagen) and random hexamer primers (Invitrogen, Karlsruhe, Germany). The reaction was allowed to proceed at 37 °C for 60 min and cDNA products were stored at -20 °C until use. For amplification of rat CysC, aliquots of first strand cDNAs were subjected to PCR with primers 5'-AGT ACA ACA AGG GCA GCA ACG ATG-3' and 5'-AGG AGA AGA GAA CCA GGG GAC AGC-3', dNTPs (each 10 mmol/L dATP, dCTP, dGTP, dTTP) in 1 \times PCR

reaction buffer and 2.5 U *Taq* DNA polymerase (Roche Molecular Biochemicals, Mannheim, Germany) resulting in the amplification of a 454-bp CysC fragment^[19]. PCR conditions were at 94 °C for 5 min (initial denaturation), at 94 °C for 60 s, at 60 °C for 60 s, at 72 °C for 3 min (40 cycles), and at 72 °C for 10 min (final extension). Aliquots of the reaction products were separated and visualized on 1% (w/v) agarose gels and the identity of the fragment was demonstrated by sequencing. Quantitative analysis of rat CysC mRNA was performed with a LightCycler (LC) System in 20 μ L reaction volume using the LC-FastStart DNA Master SYBR Green I kit (Roche). Thermocycling was performed with 2 μ L of cDNA products and 0.75 μ mol/L of each primer specific for CysC. No-template controls were prepared by adding 2 μ L PCR grade, sterile H₂O to 18 μ L of master mix. Cycling conditions were one cycle of denaturation at 95 °C for 10 min, followed by 45 amplification cycles at 95 °C for 10 s, at 60 °C for 5 s, at 72 °C for 22 s. The PCR products were melted in a temperature transition procedure from 65 °C to 95 °C in steps of 0.1 °C/s and fluorescence was measured and plotted online against the temperature to obtain the fragment-specific melting points. Differences between crossing points were taken to estimate the relative concentrations.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

For immunoblotting, proteins from rat liver cells were separated under reducing conditions on 4-12% Bis-Tris gradient gels using MES running buffer (Invitrogen). The electroblotting onto 0.2- μ m protran NC membranes (Schleicher & Schuell, Dassel, Germany) was carried out according to standard procedures. Blocking and incubation of the membranes with individual antibodies were performed as described previously^[18] with slight modifications. Briefly, membranes were blocked with 5% (w/v) non-fat milk powder in Tris-buffered saline-Tween 20 (TBST). For Western blot analysis, the following primary antibodies were diluted in 2.5% (w/v) non-fat milk powder in TBST: clone asm-1 (Cymbus Biotech., Chandlers Ford, UK) against α -smooth muscle actin (α -SMA) (1:10 000), rabbit anti-human CysC rat/human CysC and its precursor (1:500) (Upstate Biotechnology, Lake Placid, USA), rabbit anti-phospho-Smad2 (Ser465/467) (1:1000) (New England Biolabs GmbH, Frankfurt/Main, Germany), rabbit anti-Smad2 (1:200) (Zymed Laboratories, San Francisco, CA, USA), goat anti-Smad2/3 (N-19), (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:100), mouse anti- β -actin (1:10 000) (Cymbus). The antiserum PS1 was raised against a phosphorylated C-terminal peptide of Smad1 and showed cross-reactivity with phosphorylated Smad3 (1:1 000)^[20]. Primary antibodies were visualized using horseradish peroxidase (HRP)-conjugated anti-mouse, -rabbit or -goat IgG (Santa Cruz Biotechnology) or alkaline phosphatase (AP)-conjugated anti-rabbit or mouse IgG (Santa Cruz Biotechnology). Alternatively, the primary antibodies against CysC and Smad2/3 (N-19) were detected with swine anti-rabbit and rabbit anti-goat IgG biotin antibody followed by streptavidin-HRP conjugate (DAKO Diagnostics, Hamburg, Germany). Staining was

performed with the SuperSignal West Dura Extended chemiluminescent substrate (Pierce, Rockford, IL, USA) for HRP exposed in a Lumi-Imager™ (Roche) or a color staining using NBT/BCIP substrate (Perbio Science, Bonn, Germany).

Sequence analysis

All primers were obtained from MWG-Biotech (Ebersberg, Germany) and sequencing was done with the ABI PRISM BigDye® termination reaction kit (PE Applied Biosystems, Weiterstadt, Germany) as described^[21].

Enzyme linked fluorescence cytological labeling assay (ELF 97 assay)

Ten thousand cells per well were cultured in black 96-well plates for different times under standard conditions and fixed with 4% (w/v) paraformaldehyde in phosphate buffered saline (PBS) (pH 7.4). For generation of myofibroblasts (MFBs), HSCs cultured for seven days were trypsinized and subcultured for further 3 days. The cells were permeabilized on ice in 0.1% (w/v) Triton X-100/0.1% (w/v) sodium citrate, blocked in 1% (w/v) BSA/PBS pH 7.4 and incubated with anti- α -smooth muscle actin (1:500) or anti-CysC antibody (1:200). Non-immune IgGs were used as negative controls. The ELF-97 assay (Molecular Probes Europe; Leiden, NL) was performed according to an established procedure^[22]. The fluorescence intensity was measured in a Multilabel counter (Victor, Wallac ADL GmbH, Freiburg, Germany) for the quantitative evaluation of α -SMA and CysC (excitation 365 nm, emission 515 nm). The staining intensity of negative controls was subtracted from the determined values and in parallel the fluorescence was also recorded in an inverted fluorescence microscope (Axiovert M135, Zeiss, Frankfurt, Germany). Subsequently, to normalize the fluorescence values (FU) of α -SMA or CysC, the DNA content of parallel cultures was measured fluorometrically (excitation 485 nm, emission 525 nm) and quantified against calf thymus DNA as standard using the SYBR Green I dye (Molecular Probes Europe).

Immunofluorescence staining and confocal microscopy

Forty thousand cells per well (1.8 cm²) were cultured in 4 chamber polystyrene vessels (Becton, Dickinson, Franklin Lakes, NJ, USA) for different times under standard conditions and fixed with 4% (w/v) paraformaldehyde in PBS (pH 7.4). Permeabilization, blocking and CysC antibody (Upstate Biotechnology, 1:200) incubation were carried out as described above. After washing, a biotin-linked swine anti-rabbit IgG (DAKO) (1:300) was used as the secondary antibody followed by incubation with streptavidin-FITC (DAKO) (1:150). For the detection of α -SMA, a direct CY3-conjugated antibody (clone AC-40; Sigma, Taufkirchen, Germany) was used (1:200). Non-immune IgGs (Santa Cruz) served as negative controls. After washing and mounting with antifade, the cells were subjected to high-resolution confocal laser scanning microscopy in a LS 410 inverse microscope (Zeiss) using a standard objective (40 \times 1.3 oil).

Adenoviral infection of HSCs and luciferase measurement

Adenoviral stocks of Ad (CAGA)₉-MLP-luciferase were

prepared as previously described^[23]. Two thousand cells per well were cultured in black 96 well plates. After infection at a multiplicity of infection (MOI) of 50, cells were serum starved at 0.2% FCS for 16 h, pre-incubated with or without rhCysC for 1.5 h, respectively, followed by cytokine stimulation as indicated. The luciferase activity was measured with the Steady-Glo luciferase assay substrate (Promega, Madison, WI, USA) according to the manufacturer's protocol.

Affinity labeling and cross-linking of TGF- β receptors

Affinity labeling with [¹²⁵I]-TGF- β 1 (Amersham Pharmacia Biotech, Freiburg, Germany) and cross-linking were performed as described previously^[24]. Briefly, confluent monolayers of HSCs (5 \times 10⁵ cells/10 cm²) were cultured for 4 days in medium containing 10% FCS. After aspiration of medium, monolayers were washed with Krebs-Ringer-HEPES binding buffer (128 mmol/L NaCl, 5 mmol/L KCl, 5 mmol/L MgSO₄, 13 mmol/L CaCl₂, 50 mmol/L HEPES) containing 0.5% (w/v) BSA (radioimmunoassay grade) and pre-incubated with or without CysC for 1 h at 37 °C. Cells were washed with ice-cold buffer and receptor binding was performed in the presence or absence of CysC. After equilibration for 10 min on ice, cells were incubated with 2.8 ng/mL [¹²⁵I]-TGF- β 1 (1 621 Ci/mmol) with or without a 200-fold excess of unlabeled TGF- β 1 (R&D Systems, Minneapolis, USA) for 3 h at 4 °C. As a further control, an antibody directed against the extracellular domain of betaglycan was used to suppress ligand binding. Bound ligand was cross-linked to its receptor in Krebs-Ringer-HEPES buffer lacking the BSA with disuccinimidyl suberate (Perbio Science) for 15 min at 4 °C. The reaction was stopped by aspiration of the supernatant followed by washing with STE buffer [10 mmol/L Tris-HCl pH 7.4, 250 mmol/L sucrose, 1 mmol/L ethylenediaminetetraacetic acid (EDTA)] including 1 mmol/L phenyl methyl sulfonyl fluoride (PMSF). Cross-linked [¹²⁵I]-TGF- β 1 was extracted with TTE buffer (10 mmol/L Tris-HCl, pH 7.4, 10 mL/L (v/v) Triton X-100, 1 mmol/L EDTA) including proteinase inhibitor cocktail complete (Roche). The detergent soluble, cell debris-free fraction was precipitated by ice-cold 10% TCA (w/v) in the presence of 0.1 mg/mL sodium deoxycholate (DOC). The protein precipitates were subsequently washed with acetone, air dried, and dissolved in a small volume of LDS electrophoresis sample buffer (Invitrogen). Sample volumes corresponding to the normalized DNA contents were subjected to NuPAGE SDS-PAGE. For autoradiography, dried gels were exposed to KODAK X-OMAT AR films (Eastman Kodak Company, Rochester, NY, USA) using intensifying screens.

CysC secretion in HSCs

Serum-starved HSCs were treated with rhTGF- β 1 (10 ng/mL) for 24 or 48 h and the cell-free supernatants were subjected to standard immunoprecipitation using goat antibody (sc-16989) directed against CysC (Santa Cruz). The resulting precipitates were washed and analyzed for CysC expression by Western blotting using a primary rabbit antibody directed against CysC (Upstate) and

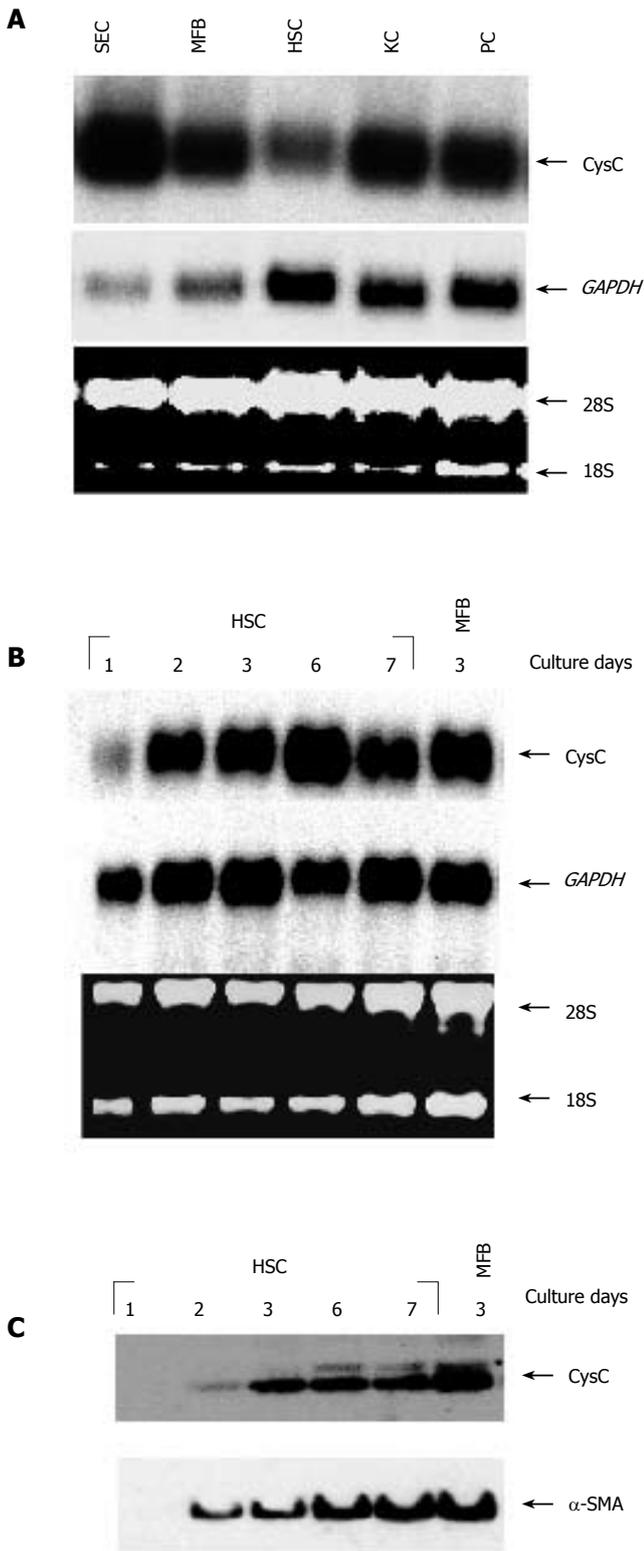


Figure 1 CysC mRNA expression in different rat liver cell types. **A:** Equal amounts of total RNA samples (10 µg) isolated from cultured SECs, MFBs, HSCs, KCs, and PCs were analyzed by Northern blot. The blot was hybridized with a ³²P-labeled probe specific for rat CysC; **B:** total RNA samples (5 µg/lane) of trans-differentiating HSCs/MFBs at indicated time intervals were analyzed for CysC expression by Northern blot analysis. As internal loading controls (**A and B**), the 18S and 28S rRNA as well as signals obtained after hybridization with a GAPDH-specific probe are shown. The blots were exposed for 4 (GAPDH) or 24 h (CysC), respectively; **C:** protein extracts (20 µg/lane) from primary rat HSCs cultured for 1 to 7 days or rat MFBs cultured for 3 days were analyzed for CysC expression by Western blotting under reducing conditions (50 mmol/L DTT) using antibody P-14 (sc-16989). To verify cellular trans-differentiation, the blot was stripped, and tested for α-SMA expression using a mouse monoclonal antibody (clone asm-1).

visualized with a goat anti-rabbit IgG-HRP conjugate.

RESULTS

Expression of CysC in isolated liver cells

To analyze the expression of CysC in different liver cell subpopulations, we performed Northern blot analysis (Figure 1A). In agreement with the general assumption that CysC is a constitutive active gene expressed in all nucleated cells at a constant rate^[25], we found a single CysC transcript of 0.8-0.9 kb in size in SECs, KCs, PCs, HSCs and their trans-differentiated myofibroblastic phenotype. However, compared to HSCs, we found a significant increase of CysC transcripts in their trans-differentiated phenotype. Subsequent analysis revealed that the activation of HSCs was accompanied with an upregulation of CysC expression during the trans-differentiation process (Figure 1B). In these analyses, the levels of mRNA encoding the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as well as the 28S and 18S rRNAs were used as internal quality and loading controls. To demonstrate CysC expression at the protein level, we further performed Western blot analysis using cellular extracts isolated from cells at different time points of trans-differentiation (Figure 1C). We found that the amount of CysC protein correlated with the amounts of transcripts. To illustrate and confirm the increase of CysC protein more quantitatively, we further performed confocal immunocytochemistry (Figure 2A) and ELF-assay (Figure 2B), respectively. Again, both visualizations of cellular CysC expression by confocal laser scanning microscopy and quantitative ELF-assay indicated that CysC expression was strongly increased during cellular activation and trans-differentiation.

CysC did not modulate TGF-β1 signal transduction in HSCs

A recent report demonstrated that CysC is able to antagonize TGF-β binding to its cell surface receptors thereby inhibiting TGF-β signaling^[9]. To test if this antagonizing effect on TGF-β signaling was also present in HSCs, we monitored the changes in the TGF-β sensitive CAGA-MLP-luciferase reporter assay system^[26]. Therefore, cultured HSCs were first infected with 50 MOI Ad-(CAGA)₉-MLP-luciferase^[22], preincubated with different concentrations of rhCysC and then exposed to TGF-β1. The TGF-β-stimulated luciferase activity was compared to unstimulated control cells, which revealed that preincubation with CysC had no effect on the reporter assay (Figure 3A). Similarly, Western blot analysis further revealed that TGF-β-induced phosphorylation of Smad2 and Smad3 did not change in the presence of CysC, again demonstrating that this cysteine protease inhibitor could not antagonize TGF-β signaling in cultured HSCs (Figure 3B).

CysC promoted TGF-β binding to TGF-β type III receptor betaglycan

Based on a recent report demonstrating that CysC could dose-dependently inhibit the physical interaction of TGF-β to murine TGF-β type II receptor^[9], we performed cross-linking experiments to test if CysC had the same

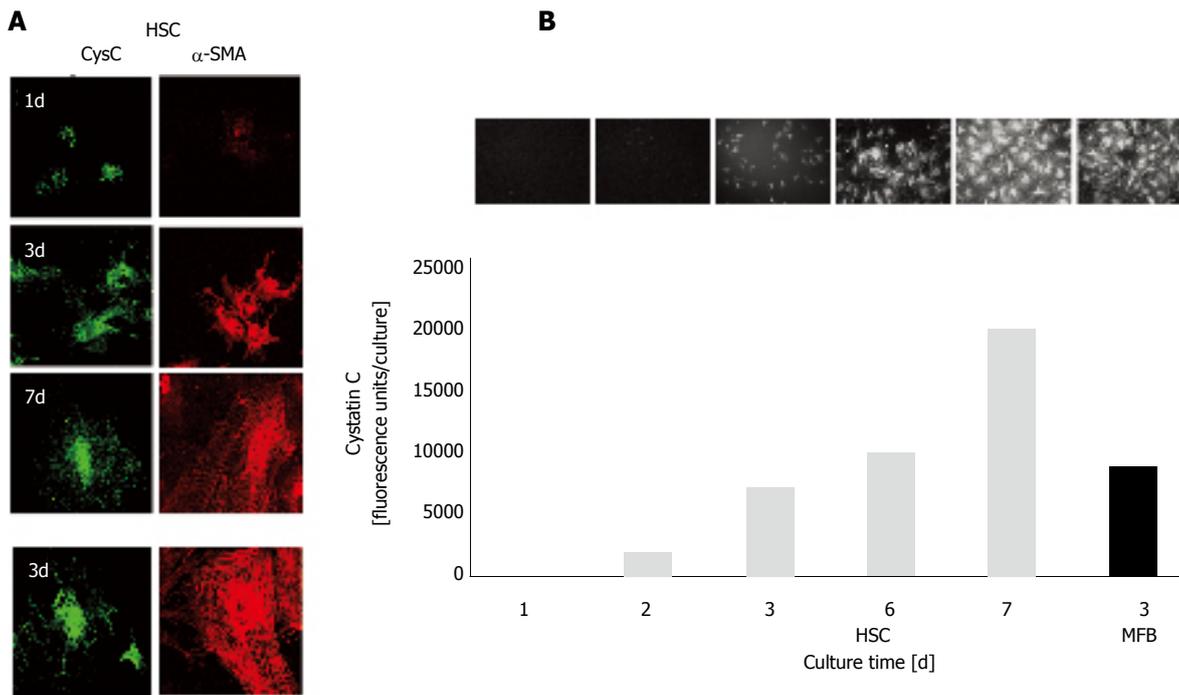


Figure 2 Confocal laser scanning microscopy and ELF assay for quantitative determination of CysC expression in HSCs and MFBs. **A:** HSCs/MFBs were seeded on glass cover slides for indicated time intervals. After fixation, cells were permeabilized and incubated with primary antibodies directed against CysC or α -SMA. Primary antibodies were visualized with a FITC-labeled secondary antibody (CysC, green fluorescence) or a Cy3-labeled secondary antibody (α -SMA, red fluorescence). Original magnification was $\times 400$. Negative controls, using normal IgGs instead of primary antibodies showed no staining (data not shown); **B:** HSCs/MFBs cultured in 96-well plates for indicated time intervals were fixed and stained for CysC. The staining was visualized by fluorescence microscopy (original magnification $\times 200$). Fluorescence intensities were measured quantitatively using a 96-well fluorescence reader (excitation 365 nm, emission ≥ 515 nm).

effects in the rat system. Interestingly, we found that the binding of TGF- β to the type II receptor was not affected in the presence of physiological concentration of CysC (data not shown). Since the transmembrane signaling by TGF- β was critically modulated in the presence of the type III receptor (betaglycan), which was expressed at high level in HSCs and in turn associated with the type II receptor in a second step, we next addressed the question if CysC was able to modify the binding of TGF- β to this receptor. Binding of TGF- $\beta 1$ to betaglycan was analyzed by cross-linking experiments using iodinated TGF- $\beta 1$. HSCs cultured for 4 days were first incubated with rhCysC. Thereafter, the cells were exposed to [125 I]-TGF- $\beta 1$ and the iodinated ligand was chemically cross-linked to the receptors. Subsequently, betaglycan was separated by SDS-PAGE, and the amount of ligand bound to this receptor was visualized by autoradiography (Figure 4, lanes 1 and 2). Interestingly, we found that the binding to betaglycan was elevated after preincubation with CysC. The specificity for the betaglycan labeling was demonstrated by preincubation with a betaglycan-specific antibody (Figure 4, lanes 3 and 4).

TGF- β and PDGF-BB suppressed CysC expression in HSCs

Several reports demonstrated that CysC transcript expression could be stimulated by TGF- $\beta 1$ ^[9,10,27]. To test if CysC expression was induced in HSCs by TGF- $\beta 1$, we performed Northern blot analysis with RNA isolated from HSCs that were treated with TGF- $\beta 1$ (5 ng/mL) for 18 or 38 h, respectively (Figure 5A, lanes 2 and 5). Surprisingly,

the contents of CysC transcripts were decreased in TGF- β -stimulated HSCs. Furthermore, the same effect was observed when PDGF-BB was applied causing a significant decrease of CysC transcript expression (Figure 5A, lanes 3 and 6). To confirm that CysC expression in HSCs was indeed suppressed by TGF- β and PDGF-BB, we repeated our experiments at the protein level. As shown in Figure 5B, both TGF- $\beta 1$ and PDGF-BB significantly suppressed the synthesis of CysC in HSCs.

TGF- β increased secretion of CysC in HSCs

To further explore the impact of TGF- β on CysC regulation, we tested if TGF- $\beta 1$ could modify CysC secretion. Therefore, we treated serum-starved HSCs with TGF- $\beta 1$ (10 ng/mL) for 24 or 48 h and determined the amount of CysC in the cell-free supernatants. Compared to control cells, the amount of secreted CysC was markedly increased in cultures stimulated with TGF- $\beta 1$ (Figure 6). Furthermore, we detected two different CysC protein bands with an approximate molecular mass of 13 and 18 ku most likely representing proteins with a different N-glycosylation status^[28].

DISCUSSION

The response to chronic liver injury is wound healing and subsequently fibrosis representing the enhanced ECM production and deposition ending in cirrhosis. In the last decades a growing body of evidence indicates that HSCs and their trans-differentiated phenotype, the MFBs, as

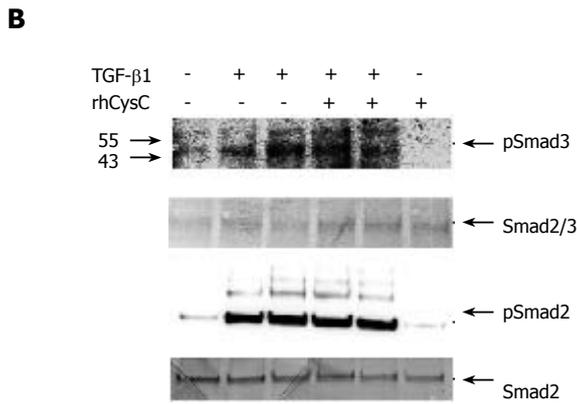
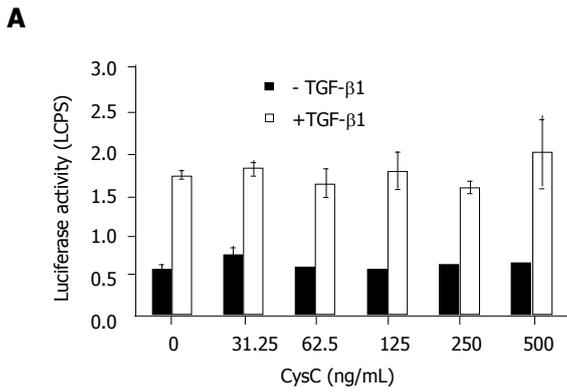


Figure 3 Effect of rhCysC on TGF-β1-induced reporter gene activity and Smad2/3 phosphorylation. **A:** Cultured HSCs were infected with 50 MOI Ad-(CAGA)₃-MLP-luciferase followed by 1 h incubation with the indicated doses of rhCysC prior to exposure with TGF-β1 (1 ng/mL) for 4 h or left untreated. The mean ±SD of the measured luciferase activities (n=4) was given; **B:** phosphorylated Smad2 (pSmad2) and Smad3 (pSmad3) were detected by immunoblotting of whole cell protein lysates prepared after treatment of HSCs with or without 500 ng/mL rhCysC for 1 h followed by incubation with 1 or 5 ng/mL TGF-β1, respectively for an additional hour. The amount of total Smad2/3 or Smad3 was used as internal loading control.

well as TGF-β1 and PDGF-BB are central cell types and mediators in this wound healing process. In healthy livers, the balance between synthesis and degradation of ECM is tightly controlled. During injury and fibrosis progression, there is increased expression of matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) resulting in an imbalance in the turnover of ECM and a conversion of the low-density subendothelial matrix into matrix-rich interstitial collagens. Additionally, the activity of collagenolytic cathepsins is elevated in experimental and clinical hepatic fibrogenesis^[29,30] and elevated serum concentrations of CysC are found in patients suffering from chronic liver diseases^[12,13]. Therefore, it is possible that an imbalance between cathepsins and CysC contributes to liver fibrosis. The demonstration that members of the cystatin superfamily can physically interact with metalloproteinases thereby protecting them from autolytic activation, further suggesting that there is a direct linkage between the MMP/TIMP and the cathepsin/cystatin enzyme systems^[31]. Although all these studies have clearly shown that elevated serum CysC concentrations are clinically important for the progression of liver fibrogenesis, the exact cellular source and the regulation

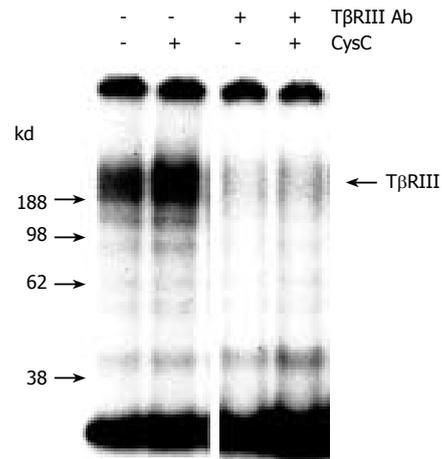


Figure 4 Effect of rhCysC on TGF-β1 binding to beta-glycan. Cultured HSCs were incubated for 1 h with or without rhCysC or an antibody specific for the extracellular domain of beta-glycan followed by the application of [¹²⁵I]-TGF-β1. The ligand was chemically cross-linked to the receptor and subsequently TβRIII was immunoprecipitated, separated by SDS-PAGE, and visualized by autoradiography. Control experiments including a 200-fold excess of unlabeled TGF-β1 showed no receptor labeling (data not shown).

of CysC within different liver cell subpopulations have not been explored.

In the present study, all liver cell types tested were able to synthesize CysC, CysC expression was up-regulated in activated HSCs and trans-differentiated MFBs, TGF-β1 and PDGF-BB suppressed CysC expression at the RNA and protein level, CysC did not modulate TGF-β1 signal transduction in HSCs but induced affinity of betaglycan for TGF-β1, TGF-β1 induced secretion of this inhibitor of cysteine proteinases.

The finding that all liver cells tested express CysC (Figure 1A) is not surprising since it has been well established that CysC behaves like a “housekeeping gene” expressed in all nucleated cells, in all tissues and cell types, although mRNA levels vary several-fold between and among the tissues^[25]. However, the finding that CysC expression is strongly increased in activated HSCs and trans-differentiated MFBs is somewhat unexpected since previous studies have demonstrated that the CysC promoter is of constitutive nature^[32]. This upregulation is consistent with the findings that patients with liver cirrhosis have an average three-fold greater serum CysC concentration which closely matches to the degrees of fibrosis and is elevated even in patients with mild fibrosis. Thus, increased CysC levels in serum of these patients are at least partially due to a higher expression of this protein inhibitor of cysteine proteinases. The observation that both TGF-β and PDGF-BB could suppress the expression of CysC is at first somewhat striking and unexpected since TGF-β1 and PDGF-BB are the main effectors during hepatic fibrogenesis and, moreover, TGF-β1 can stimulate CysC expression in murine embryo cells, uterine decidual and 3T3-L1 cells^[9,10,27]. The conflict might be explained by the finding that only HSCs respond to TGF-β1, whereas fully trans-differentiated MFBs are insensitive to the treatment with TGF-β1^[24]. Thus, in ongoing hepatic fibrogenesis, when HSCs become refractory against

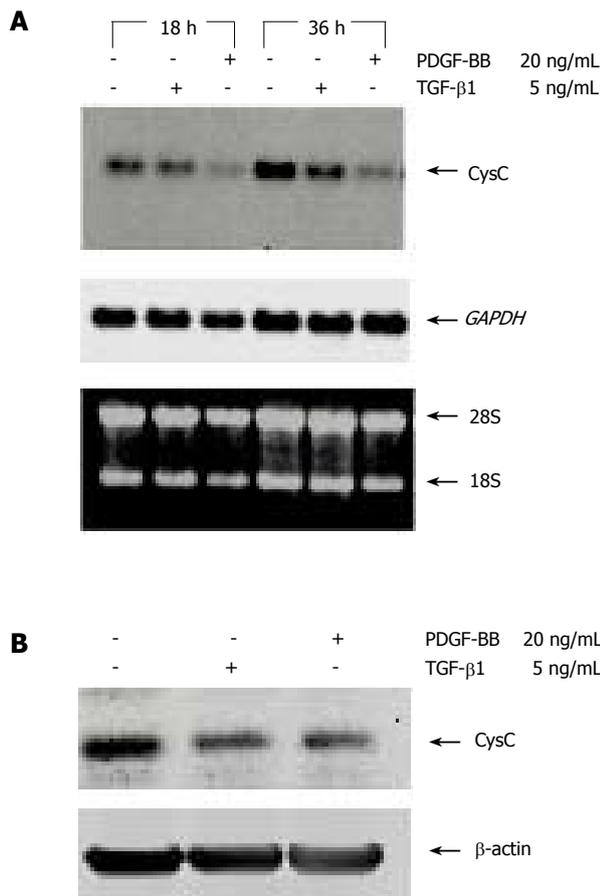


Figure 5 Effect of PDGF-BB and TGF- β 1 on CysC expression in HSCs. **A:** Cultured HSCs were serum starved to 2 mL/L FCS followed by treatment with or without PDGF-BB or TGF- β 1 for 18 and 36 h, respectively. Total RNA (5 μ g/lane) was isolated and analyzed by Northern blot for CysC expression. As internal loading controls, the 18S and 28S rRNA as well as signals obtained after hybridization with a GAPDH-specific probe were shown; **B:** for CysC protein expression, cells were treated with or without PDGF-BB or TGF- β 1 for 72 h and protein lysates (20 μ g/lane) were subjected to Western blot analysis using an antibody specific for CysC. The expression of β -actin was used as internal protein loading control.

TGF- β , they start to increase CysC expression potentially leading to the observed higher serum concentrations.

In HSCs, TGF- β signaling is not influenced by the presence of CysC. Although CysC has been found to increase the ligand binding to betaglycan, we observed that it had no effects on TGF- β receptor type II binding (data not shown) and TGF- β reporter gene assay or Smad2/3 phosphorylation. Possibly, CysC inhibits a specific proteinase required for shedding of betaglycan. In line with such a hypothesis, the shedding of betaglycan is mediated by membrane type MMP-1^[33] and members of the cystatin superfamily can interact with MMPs and protect them from autolytic degradation^[31]. The missing impact of CysC on TGF- β -binding to the type II receptor might be due to the absence of specialized spliced forms of the type II receptor, i.e. type IIb present in mice^[34] but not in rat HSCs/MFBs (Meurer, Gressner, Weiskirchen, unpublished observation).

Finally, we found that TGF- β induced secretion of CysC in HSCs. This ability of TGF- β is also described in

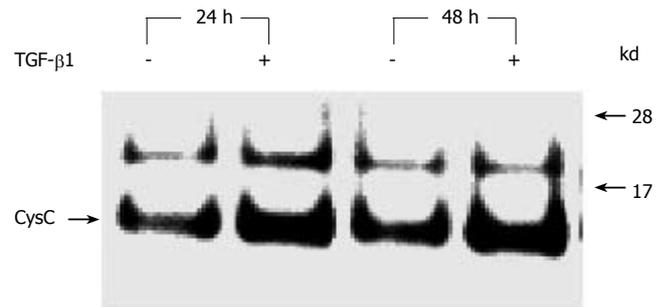


Figure 6 TGF- β 1-induced CysC secretion in HSCs. Serum-starved HSCs were treated with TGF- β 1 (10 ng/mL) for 24 or 48 h and the cell-free supernatants were subjected to immunoprecipitation using goat antibody P-14 directed against CysC. Subsequently, the precipitates were analyzed for CysC expression by Western blotting using an independent primary rabbit antibody directed against CysC and visualized with a goat anti-rabbit IgG-HRP conjugate.

vascular smooth muscle cells^[8], suggesting that it might be a general hallmark of smooth muscle cells. It is also conceivable that the observed decrease of cellular CysC content upon stimulation with TGF- β 1 is a consequence of CysC release from intracellular storage sites. However, this effect might only in part contribute to the intracellular decrease of CysC since we observed a clear decrease of CysC mRNA in cells stimulated with TGF- β 1.

In conclusion, the concentration of serum CysC is correlated with the severity of liver dysfunction. CysC expression is upregulated in the course of HSC activation and trans-differentiation into MFB, which gives a plausible explanation for the correlation of CysC concentrations with the progression of liver fibrosis.

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Microproteinuria in patients with inflammatory bowel disease: Is it associated with the disease activity or the treatment with 5-aminosalicylic acid?

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CONCLUSION: Microproteinuria is mainly associated with UC and its activity but not affected by 5-ASA.

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Key words: Inflammatory bowel disease; Microproteinuria; 5-aminosalicylic acid

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Abstract

AIM: To investigate whether microproteinuria in patients with inflammatory bowel disease (IBD) is associated with the disease activity or the treatment with 5-aminosalicylic acid (5-ASA).

METHODS: We prospectively studied microproteinuria in 86 consecutive patients with IBD, 61 with ulcerative colitis (UC) and 25 with Crohn's disease (CD), before as well as 2 and 6 months after their inclusion in the study. Forty-six patients received 5-ASA for a period of 28.8 months (range 1-168 mo). Microalbuminuria (mALB) and urine levels of the renal tubular proteins β_2 -microglobulin (β_2 mGLB) and β -N-acetyl-D-glucosamidase (β -NAG) as well as the creatinine clearance were determined in a 12-h overnight urine collection. Tumor necrosis factor- α (TNF- α) serum levels were also measured.

RESULTS: A total of 277 measurements (194 in UC patients and 83 in CD patients) were performed. The prevalence of abnormal microproteinuria in UC and CD patients was 12.9% and 6.0% for mALB, 22.7% and 27.7% for β_2 mGLB, and 11.3% and 8.4% for β -NAG, respectively. mALB was not associated with IBD activity. β_2 mGLB and β -NAG urine levels were correlated to UC activity (UCAI: $P < 0.01$; UCEI: $P < 0.005$). mALB in UC patients and β -NAG urine levels in CD patients were related to TNF- α serum levels. An association was noticed between microproteinuria and smoking habit. Treatment with 5-ASA was not correlated to the severity of microproteinuria or to the changes of creatinine clearance.

INTRODUCTION

Complications of the urinary system are not uncommon in patients with inflammatory bowel disease (IBD). Their incidence has been reported to vary from 4% to 23% and is greater in patients with more severe and long-standing disease^[1]. Apart from secondary complications, such as nephrolithiasis, hydronephrosis and amyloidosis, other associations between IBD and renal disorders have also been described, regardless of treatment^[2-5]. Membranous glomerulonephritis, rapidly progressive glomerulonephritis, mesangiocapillary glomerulonephritis, IgA nephropathy, thin basement membrane disease and kidney granuloma are connected with IBD^[6-10]. In addition, non-specific morphological changes in the glomeruli of patients with IBD, such as podocyte effacement and mesangial deposition of immunoglobulin and complement have also been well documented^[11]. On the contrary, cases of interstitial nephritis are attributed to the nephrotoxic effect of aminosalicylates^[11]. However, before the establishment of 5-ASA as a treatment of choice in IBD, studies have identified renal tubular lesions in a proportion of patients^[12]. Till date, more than 37 cases implicating the drug in interstitial nephritis (mainly mesalazine but also sulfasalazine, balsalazide, and olsalazine) have been reported to the Committee on Safety of Medicines (CSM)^[13].

Abnormal microproteinuria [microalbuminuria (mALB) and/or tubular proteinuria] has been reported both in patients with ulcerative colitis (UC) and in those with Crohn's disease (CD)^[13-17]. Increased mALB, equivalent to an albumin excretion rate of 20-200 $\mu\text{g}/\text{min}$, is generally regarded as a sensitive indicator of glomerular disease and has been widely used as a clinical marker of incipient diabetic nephropathy^[18]. Mahmud *et al.*^[14] have found that there is a significant correlation between mALB and IBD activity. Increased urine levels of some tubular proteins, such as β -N-acetyl-D-glucosamidase (β -NAG), α ₁-microglobulin, β ₂-microglobulin (β ₂mGLB), alkaline phosphatase and gamma-glutamyltransferase, have been reported to be reliable indirect indices of renal tubular dysfunction^[19]. Moreover, Riley *et al.*^[15] have also found that the incidence of elevated urinary β -NAG is low in patients with quiescent UC, which is irrelevant to the dose and duration of mesalazine treatment. Kreisel *et al.*^[17] reported that there is a strong correlation between abnormal tubular proteinuria and the activity of UC but not the activity of CD. On the contrary, Schreiber *et al.*^[20] showed that increased prevalence of tubular proteinuria is attributed to high dosages of 5-ASA.

The etiology of abnormal microproteinuria in patients with IBD remains ambiguous. We conducted the present 6-month prospective study in order to investigate the incidence of mALB and tubular proteinuria in patients with IBD, emphasizing on the possible relationship between microproteinuria and the disease activity or the treatment with 5-ASA.

MATERIALS AND METHODS

Patients and sample selection

A total of 86 out of 166 screened consecutive Caucasian patients with confirmed chronic IBD (61 with UC and 25 with CD), treated at the Gastroenterology Department of the Red Cross Hospital of Athens from June 1998 to April 2001, were prospectively included in this study. Patients with signs of urinary tract infection and a history of acute or chronic nephropathy, diabetes mellitus, hypertension, severe liver disease, cardiac failure, use of non-steroidal anti-inflammatory drugs (NSAIDs) or other nephrotoxic drugs, patients with a present or recent pregnancy, patients treated with olsalazine, and patients who refused to participate in this study were excluded. Fifty patients were male, 36 were female and their mean age was 42.7 (range 15-77 years) years. Fifty-nine were out-patients and 27 were in-patients. At the time of their inclusion in this study, the IBD was active in 38 patients (27 with UC and 11 with CD) and inactive in 48 patients. Thirty out of eighty-six patients were first diagnosed with IBD (23 with UC and 7 with CD). The mean duration of the disease was 69.8 months (range 0-576 months). The group of patients with UC consisted of 16 patients with total colitis and 45 with left sided colitis (two of them with rectal disease only). The group of patients with CD included 11 patients with ileo-colonic disease, 8 with small bowel disease only and 6 with colonic disease only. Due to the small number of patients, two subgroups of patients with CD were considered in the subsequent analysis, one consisted of the

patients with ileo-colonic or colonic disease and the other consisted of those suffering from small bowel disease only. Extra-intestinal manifestations at present or during the last 2 months before the first meeting or during follow-up, were recorded in 29 patients (14 with UC, 15 with CD). Forty-six patients received mesalazine (Salofalk 2-3 g/day or Asacol 1.6-2.4 g/day) for a mean period of 28.8 months (range 1-168 months). During the last year, 20 of them were treated with prednisolone for a mean period of 1.9 months (range 1-12 months) and 6 with azathioprine for a mean period of 4.9 months (range 1-10 months).

According to the study design, all patients were evaluated at the time of their inclusion in the study (stage 1), two (stage 3) and six (stage 4) months afterwards. Twenty-seven patients (19 with UC, 8 with CD) with severely exacerbated disease at the time of their inclusion in the study were re-evaluated 10 days after intensive corticosteroid-based treatment (stage 2). A meticulous routine and more specific clinical and laboratory evaluation of the patients was conducted in each stage of the study, including determination of the hematocrit value, white blood cell and platelet count, serum levels of C-reactive protein (CRP) and tumor necrosis factor-alpha (TNF- α), usual urinalysis, levels of mALB and tubular proteinuria (β ₂mGLB, β -NAG) and creatinine clearance. Measurements of microproteinuria and creatinine clearance were performed using a 12-h urine collection, which has been proved equivalent to a 24-h urine collection^[21] or an 8-h urine collection^[22]. The criterion of our choice was the better compliance of our patients. The clinical disease activity quantifications were performed on the day of urine collection, using reliable activity indices for UC^[23] and CD^[24]. All patients with UC underwent colonoscopy at the 1st, 3rd, and 4th stages of the study and an endoscopic index of the activity of UC (UCEI)^[25] was calculated for these patients^[25]. Finally, a detailed drug history was taken from all the patients to confirm the type, dose and duration of medication.

The blood samples for the measurement of serum levels of TNF- α after centrifugation were stored at -48 °C. The urine samples for the determination of urine levels of microalbumin were stored at 2-8 °C for a time not exceeding 2 weeks, at -20 °C for β ₂mGLB and at -48 °C for β -NAG, respectively.

Microproteinuria (mALB and tubular proteinuria) was correlated to patients' characteristics including sex, age, smoking habits; features of the diseases including activity, extent, duration, presence of extraintestinal manifestations; medication including type (mesalazine, corticosteroids, azathioprine) and duration. Furthermore, separate analyses of the courses of microproteinuria were conducted for patients with highly active disease at the time of their inclusion in the study and those with a first diagnosis of the disease, who did not receive any medication.

Analytical techniques (chemical analysis)

Urea and creatinine in serum as well as creatinine in urine were measured with standard techniques. Serum CRP concentrations were determined by nephelometry.

Urinary albumin concentrations were measured using a radioimmunologic assay (¹²⁵I radioimmunologic analysis, EURO/DPC, Glyn Rhonwy, UK), which could indicate

Table 1 Incidence of abnormal microproteinuria in patients with active and inactive IBD (%)

| | Ulcerative colitis (n = 194) | | | Crohn's disease (n = 83) | | |
|---|------------------------------|--------------------------------|--------|-------------------------------|---------------------------------|-------|
| | Active (n = 49) UCAI ≥ 6 | Inactive (n = 145) UCAI < 6 | P | Active (n = 15) CDAI ≥ 150 | Inactive (n = 68) CDAI < 150 | P |
| Urine m-ALB (>18 µg/min) | 16.3 | 11.7 | NS | 13.3 | 4.4 | 0.06 |
| Urine β ₂ mGLB (>120 µg/min) | 40.8 | 16.6 | <0.001 | 53.3 | 22.1 | <0.05 |
| Urine β-NAG (>5.9 U/L) | 26.5 | 6.2 | <0.001 | 13.3 | 7.4 | NS |

Table 2 Microproteinuria and creatinine clearance in active and inactive IBD (mean±SE)

| | Ulcerative colitis (n = 194) | | | Crohn's disease (n = 83) | | |
|------------------------------------|------------------------------|--------------------|--------|--------------------------|-------------------|-------|
| | Active (n = 49) | Inactive (n = 145) | P | Active (n = 15) | Inactive (n = 68) | P |
| Urine m-ALB (µg/min) | 11.89±3.19 | 9.0±1.03 | NS | 8.59±2.65 | 7.02±1.07 | NS |
| Urine β ₂ mGLB (µg/min) | 204±50.2 | 88.0±10.28 | <0.01 | 359±130.9 | 92.2±16.26 | <0.05 |
| Urine β-NAG (U/L) | 4.69±0.64 | 2.39±0.16 | <0.001 | 3.07±0.90 | 2.36±0.25 | NS |
| Urine Kcr (mL/min) | 114.4±6.03 | 120.3±3.24 | NS | 135.6±8.34 | 127.7±3.96 | NS |

NS: Not significant.

subtle increases in albumin excretion above the normal range but below that was detected by standard “dipstick” analysis (equivalent to a urinary albumin excretion rate of 20–200 µg/min)^[26].

Urinary β₂mGLB concentrations were measured using a radioimmunologic assay (¹²⁵I radioimmunologic analysis, RADIM)^[27].

Urinary β-NAG concentrations were determined colorimetrically using a commercially available kit (Boehringer-Mannheim, Germany). The optical density of the samples was measured at 580 nm (Cobas Bioanalyser, Roche). Results were calculated by reference to a standard curve^[15,28].

Serum TNF-α was measured using a sandwich enzyme-linked immunosorbent assay (ELISA; Quantikine, R&D Systems Inc., Minneapolis, MN, USA: sensitivity 0.35 pg/mL)^[29,30].

Quantification of disease activity

The activity of UC was quantified using the UC activity index (UCAI) introduced by Rachmilewitz^[23]. A score greater than or equal to six was considered to be suggestive of active UC. The activity of CD was estimated according to the Best CD activity index (CDAI)^[24]. CD was considered to be active in cases with CDAI greater than 150. Finally, the endoscopic activity of UC was also evaluated according to a reliable endoscopic index established by other researchers^[25].

Statistical analysis

The Mann-Whitney *U*-test, the Wilcoxon rank-sum *W*-test

and the Spearman's rank correlation test were used for statistical evaluation of the non-parametric data. Student's *t*-test was applied for statistical analysis of all parametric data. *P*<0.05 was considered statistically significant.

RESULTS

Eighty-two out of eighty-six patients participating in three stages of the study (time: 0, 2nd, and 6th month) completed the follow-up. Twenty-seven of them participated in one more stage (time: 10th day). Two out of eighty-six patients participated in two stages (time: 0, 10th day) and two in one stage only (time: 0). A total of 277 distinct evaluations were performed in 86 IBD patients, 194 in patients with UC and 83 in patients with CD. The IBD was active in 38 out of 86 patients at the 1st stage, in none out of 27 patients at the 2nd stage, in 7 out of 82 patients at the 3rd stage and in 6 out of 82 patients at the 4th stage of the study.

Abnormal urine levels of albumin (>18 µg/min), β₂mGLB (>120 µg/min) and β-NAG (>5.9 U/L) were found in 12.9%, 22.7%, and 11.3% of the patients with UC and in 6%, 27.7%, and 8.4% of those with CD, respectively, more frequently in active than in inactive disease (Table 1). The mean values of mALB, tubular microproteinuria and creatinine clearance in active and inactive IBD are shown in Table 2. No differences in albuminuria were found between active or inactive UC and CD. In contrast, significantly higher urine levels of β₂mGLB were detected both in patients with active UC and in those with active CD. Urine levels of β-NAG were

Table 3 Correlation of microproteinuria and creatinine clearance of IBD patients after 5-ASA treatment (mean±SE)

| | Ulcerative colitis (n = 194) 5-ASA treatment | | P | Crohn's disease (n = 83) 5-ASA treatment | | P |
|--|---|---------------|-------|---|--------------|-------|
| | No (n = 37) | Yes (n = 157) | | No (n = 15) | Yes (n = 68) | |
| Urine m-ALB (µg/min) | 8.99±2.10 | 9.90±1.29 | 0.794 | 8.08±2.57 | 7.13±1.08 | 0.562 |
| Urine β ₂ mGLB (µg/min) | 163±61.16 | 107±12.07 | 0.852 | 274±129.2 | 111.0±20.15 | 0.083 |
| Urine β-NAG (U/L) | 4.22±0.82 | 2.67±0.18 | 0.118 | 2.79±0.91 | 2.42±0.26 | 0.845 |
| Urine Kcr (mL/min) | 117±7.25 | 119±3.11 | 0.987 | 130±7.62 | 129±4.05 | 0.713 |

Table 4 Correlation between microproteinuria in patients with ulcerative colitis and disease activity parameters (n = 194 measurements)

| | | Ulcerative colitis activity parameters | | | | | |
|--|----------------|--|--------|--------|--------|-----------|-------------|
| | | UCAI | UCEI | CRP | ESR | Platelets | Serum TNF-α |
| Urine m-ALB (µg/min) | r _s | 0.0283 | 0.0420 | 0.0232 | 0.0091 | 0.0826 | 0.1665 |
| Urine β ₂ mGLB (µg/min) | P | 0.6954 | 0.5612 | 0.7479 | 0.9001 | 0.2520 | 0.0203 |
| Urine β-NAG (U/L) | r _s | 0.1957 | 0.1037 | 0.1059 | 0.0033 | 0.0540 | 0.0123 |
| | P | 0.0062 | 0.1501 | 0.1414 | 0.9634 | 0.4543 | 0.8648 |
| | r _s | 0.2633 | 0.2335 | 0.1438 | 0.1224 | 0.2336 | 0.1057 |
| | P | 0.0002 | 0.0010 | 0.0455 | 0.0891 | 0.0010 | 0.1426 |

r_s: Spearman's correlation coefficient; r_p: Pearson's correlation coefficient.

higher in active UC, but not in active CD.

Serum creatinine and its clearance

The levels of serum creatinine were normal in all the patients. Although creatinine clearance values varied widely, its mean values did not differ significantly between the patients with active and those with inactive UC (114.4 ± 6.03 mL/min *v*s 120.3 ± 3.24 mL/min, *P* > 0.10) as well as between the patients with active and those with inactive CD (135.6 ± 8.34 mL/min *v*s 127.7 ± 3.96 mL/min, *P* > 0.10). Our male IBD patients showed higher creatinine clearance values than female IBD patients (UC: 134 ± 3.19 mL/min *v*s 95 ± 4.07 mL/min, *P* < 0.001; CD: 137.1 ± 5.16 mL/min *v*s 118.8 ± 4.22 mL/min, *P* < 0.05). Creatinine clearance values were inversely correlated with the age of UC patients (ulcerative colitis: r_p = -0.374, *P* < 0.001; CD: r_p = -0.164, *P* > 0.10).

In addition, creatinine clearance values were higher in smokers with UC (smokers: 127 ± 4.04 mL/min; non-smokers: 114 ± 3.81 mL/min, *P* < 0.05) and in those with CD (smokers: 137.3 ± 4.50 mL/min; non-smokers: 113.1 ± 4.50 mL/min, *P* < 0.001). Finally, no correlation of creatinine clearance values with 5-ASA treatment was observed (UC patients treated with and without 5-ASA: 119 ± 3.11 mL/min and 117 ± 7.25 mL/min, respectively,

P = 0.987; CD patients treated with and without 5-ASA: 129 ± 4.05 mL/min and 130 ± 7.62 mL/min, respectively, *P* = 0.713).

Microproteinuria and IBD patients' parameters

No correlation was found between microproteinuria (mALB or tubular proteinuria) and the age or the sex of our IBD patients (data not shown). However, albuminuria was significantly lower in smokers with UC than in non-smokers (smokers: 8.22 ± 2.16 µg/min; non-smokers: 10.6 ± 1.23 µg/min, *P* = 0.021) and the urine levels of β-NAG were significantly lower in non-smokers with CD than in smokers (non-smokers: 1.62 ± 0.19 U/L; smokers: 2.93 ± 0.37 U/L, *P* = 0.023).

Microproteinuria and IBD activity

In our patients with UC, urinary concentrations of β-NAG were significantly correlated with the UCAI (r_s = 0.26, *P* < 0.0005), the UCEI (r_s = 0.23, *P* < 0.005) and the serum CRP levels (r_s = 0.14, *P* < 0.05). Similarly, in these patients urinary levels of β₂ mGLB were significantly associated with the UCAI (r_s = 0.2, *P* < 0.01) but not with the UCEI or the serum CRP levels (Table 4). On the contrary, the patients with CD did not show any correlation of tubular microproteinuria with the parameters of their disease activity (Table 5).

No significant relationship was found between the levels of mALB and the disease activity in patients with UC neither in those with CD (Tables 4 and 5). A significant association between mALB and serum levels of TNF-α was noticed only in UC patients. Finally, a history of recent or present extra-intestinal manifestations did not influence the levels of mALB and tubular microproteinuria in IBD patients (data not shown).

Microproteinuria and IBD duration and extent

A significant inverse relationship was found between the levels of mALB and the duration of UC (r_s = -0.24, *P* < 0.001). In contrast, a tendency towards a positive association was noticed between the severity of mALB and the duration of CD (r_s = 0.19, *P* = 0.078). No significant differences were found between the levels of microproteinuria (mALB and tubular microproteinuria)

Table 5 Correlation between microproteinuria in patients with Crohn's disease and disease activity parameters (*n* = 83 measurements)

| | | Crohn's disease activity parameters | | | | |
|------------------------------|-------|-------------------------------------|--------|--------|-----------|---------------------|
| | | CDAI | CRP | ESR | Platelets | Serum TNF- α |
| Urine | r_s | 0.1186 | 0.0326 | 0.0352 | 0.1483 | 0.1537 |
| m-ALB | | | | | | |
| ($\mu\text{g}/\text{min}$) | P | 0.2856 | 0.7686 | 0.7522 | 0.1810 | 0.1654 |
| Urine | r_s | 0.0813 | 0.0187 | 0.0624 | 0.1727 | 0.2100 |
| $\beta_2\text{mGLB}$ | | | | | | |
| ($\mu\text{g}/\text{min}$) | P | 0.4648 | 0.8669 | 0.5751 | 0.1184 | 0.0568 |
| Urine | r_s | 0.1106 | 0.0319 | 0.0947 | 0.0527 | 0.2676 |
| $\beta\text{-NAG}$ | | | | | | |
| (U/L) | P | 0.3196 | 0.7744 | 0.3347 | 0.6359 | 0.0145 |

r_s : Spearman's correlation coefficient; r_p : Pearson's correlation coefficient.

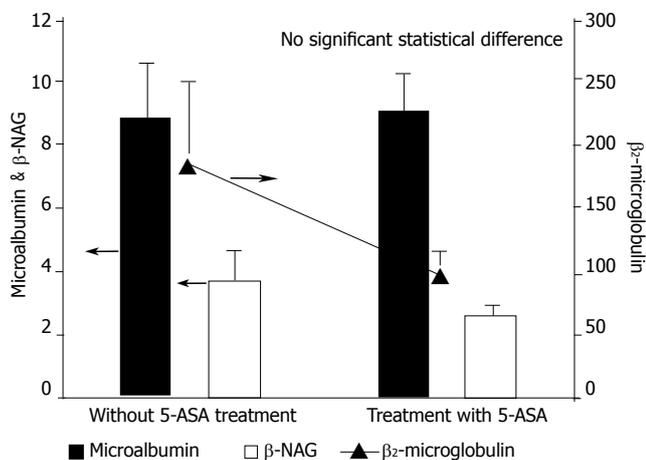


Figure 1 Correlation of IBD patients' microproteinuria (mALB, $\beta_2\text{mGLB}$, $\beta\text{-NAG}$) with 5-ASA treatment.

and the extent of UC (total *vs* left side colitis) as well as the extent of CD (small intestine+colon *vs* small intestine only).

Microproteinuria and IBD therapy

No differences were found in the levels of mALB and tubular microproteinuria between the IBD patients who received or did not receive 5-ASA therapy (Table 3 and Figure 1). Moreover, no differences were found between the duration of 5-ASA treatment and the levels of mALB (UC: $r_s = -0.06$, $P = 0.43$; CD: $r_s = 0.05$, $P = 0.70$), the urinary levels of $\beta_2\text{mGLB}$ (UC: $r_s = 0.06$, $P = 0.42$; CD: $r_s = 0.17$, $P = 0.16$) and $\beta\text{-NAG}$ (UC: $r_s = -0.02$, $P = 0.77$; CD: $r_s = 0.02$, $P = 0.88$). The levels of microproteinuria were also similar in the patients treated and those not treated with corticosteroids and/or azathioprine (data not shown).

Microproteinuria in the first-diagnosed patients with active IBD

Among the 30 IBD patients with a first diagnosis of the

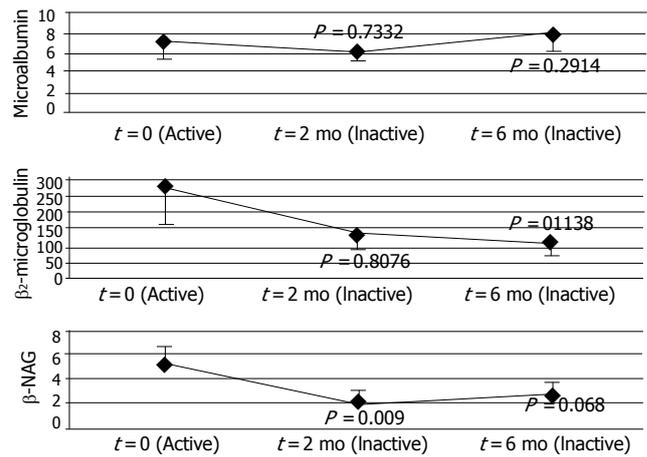


Figure 2 Changes of microproteinuria in the first-diagnosed patients with active IBD (*n* = 22) before and after inducing remission.

disease at the time of their inclusion in this study, 22 had active IBD (17 had UC, 5 had CD). The 6-month course of mALB and tubular microproteinuria in the patients with an initially active IBD never treated with 5-ASA, exhibited a particular interest (Figure 2). In these patients, microproteinuria showed a tendency of gradual reduction by the time when IBD remission was achieved, although this reduction was statistically significant for $\beta\text{-NAG}$ only.

Microproteinuria in patients with severely active IBD

Twenty-seven of the patients (19 with UC, 8 with CD) were admitted to our hospital due to severely active disease. In these patients, we also evaluated the levels of microproteinuria in one more stage 10 days after the initiation of an intensive therapy (including corticosteroids). An early (10 days after) reduction of mALB ($10.56 \pm 2.27 \mu\text{g}/\text{min}$ *vs* $6.13 \pm 1.43 \mu\text{g}/\text{min}$, $P < 0.01$) as well as urine levels of $\beta\text{-NAG}$ ($5.60 \pm 1.10 \text{ U/L}$ *vs* $3.17 \pm 0.60 \text{ U/L}$, $P < 0.05$) and $\beta_2\text{mGLB}$ ($241.2 \pm 96.82 \mu\text{g}/\text{min}$ *vs* $99.6 \pm 33.33 \mu\text{g}/\text{min}$, $P > 0.10$) was observed.

DISCUSSION

Although the incidence of renal complications is relatively low in patients with IBD, none of our patients developed clinically significant renal disease. No significant changes in serum creatinine and its clearance were recorded during the 6-month follow-up period. Furthermore, creatinine clearance was not correlated with the type, activity or the extent of IBD, which is in accordance with the majority of the published studies^[13-17].

We observed a moderately abnormal mALB in 12.9% and 6% of our patients with UC and CD, respectively, which is in agreement with Fraser *et al*^[13], Riley *et al*^[15], Bonnet *et al*^[31] and Zehnter *et al*^[32]. Increased mALB reliably predicts diabetic nephropathy and an increased incidence of cardiovascular-related morbidity and mortality in diabetic and hypertensive patients as well as in the elderly^[18,33,34]. Moreover, mALB has been described as a non-specific marker for acute illness^[35], most probably as a result of the acute phase response to inflammatory

mediators. It has been suggested that mALB in IBD patients may result from increased renal microvascular permeability in response to increased circulating cytokines^[14,30,56]. This hypothesis is compatible with our finding of a significant relationship between mALB and serum TNF- α levels in our UC patients ($P < 0.05$), a thesis which is also supported by other researchers^[13,15,31,32].

We did not find any significant correlation between the levels of mALB and the IBD activity, which is in agreement with many published studies^[13,15]. In contrast, Mahmud *et al.*^[14,37] found that increased mALB in the vast majority of IBD patients is related to both clinical and histopathological activities of the disease. Differences between these studies may be attributed to the varied disease activity. In our study, a relatively small proportion of the patients had a considerable disease activity.

Abnormal tubular microproteinuria, reflecting sub-clinical tubular damage, is a quite common finding among IBD patients^[15,17]. However, its incidence varies widely^[13-17,31,32,38], from 0% in the studies by Biddle *et al.*^[38] (β -NAG and β 2mGLB) and Mahmud *et al.*^[14] (glutathione-S-transferase) to 75% in the study by Bonnet *et al.*^[31] (γ -glutamyl transferase and alkaline phosphatase). Hitherto, it has been difficult to prove whether tubular microproteinuria results from the effect of 5-ASA or is an extra-intestinal manifestation of the IBD itself^[13]. Its structural similarity to other salicylates and non-steroidal anti-inflammatory drugs has led to the speculation that 5-ASA may cause tubulointerstitial damage of the kidney^[11].

A significant correlation between tubular microproteinuria and IBD activity was found only in our patients with UC. This might be due to the fact that our patients with severely active CD were much fewer than those with UC. Kreisel *et al.*^[17] have observed an increased urinary β -NAG excretion in 28% of the patients with UC and in 19% of those with CD, especially higher in active disease. Schreiber *et al.*^[28] acknowledged their inability to determine whether high levels of tubular microproteinuria are attributable to the severity of the disease's activity or 5-ASA treatment (or both), since 11% of their patients not receiving 5-ASA also had a tubular microproteinuria. Fraser *et al.*^[13] found that urinary excretion of β -NAG and α 1-microglobulin was increased in 48% and 52% of patients with IBD at diagnosis, respectively. A positive relationship between the urine levels of several tubular proteins and the IBD activity has been proposed by the majority of the investigators^[13-17,31,39]. In contrast, Zehnter *et al.*^[32] showed that increased urine levels of β 2-mGLB, alanine aminopeptidase and β -NAG in IBD patients were not correlated with the disease activity.

In this study, 22 first-diagnosed patients with active IBD had increased levels of tubular microproteinuria at diagnosis, which decreased after treatment with corticosteroids and 5-ASA. Moreover, 27 IBD patients with severely active disease manifested an early significant decrease of both microalbuminuria and tubular microproteinuria after being treated with corticosteroids. In addition, we found that there was a positive relationship between microalbuminuria and serum TNF- α levels, as well as between tubular microproteinuria and serum CRP

and TNF- α levels, suggesting that microproteinuria in IBD patients may be related to a systemic inflammatory response and that it is an extra-intestinal manifestation of the active disease rather than a consequence of a subclinical renal injury due to 5-ASA treatment^[13,15,17,20,31,32,37]. However, We did not find any differences in the severity of microproteinuria between the IBD patients with and those without a recent or present extra-intestinal manifestation of the disease, which is in agreement with Kreisel *et al.*^[17] and Mahmud *et al.*^[30].

Activation of the intestinal immune system, production of inflammation mediators or imbalance between activating and suppressing mediators play a pivotal role in the pathogenesis of IBD. In particular, TNF- α may play an important role in the inflammatory cascade of IBD and increased serum TNF- α levels have been reported in active IBD^[40]. It has been reported that interleukin-1 and TNF- α inhibit the synthesis and induce a shedding of cell surface glycosaminoglycans^[41], which might lead to an increased microvascular permeability and tissue damage^[36,42].

No relationship was found between the severity of microproteinuria and the extent as well as the duration of IBD in our study, which is in concordance with other studies^[14,20,32,43]. It is worth emphasizing on our finding of a significant correlation between microproteinuria and the smoking habit, which was negative in UC and positive in CD, because smoking alters the colonic mucosal blood flow^[44] and decreases its permeability. Epidemiologic data suggest that smoking exerts a protective effect against UC^[45] and is harmful to CD^[45,46]. Consequently, differences in microproteinuria between smokers with UC and those with CD may be related to these pathophysiologic and epidemiologic data.

In this study, mALB was not correlated with the IBD treatment as it is almost universally accepted^[13,15,31,32,37]. In addition, using sensitive markers of renal tubular toxicity^[19], we were unable to find any evidence that treatment with mesalazine, corticosteroids or azathioprine was related to an increased tubular damage. However, this lack of correlation does not necessarily exclude nephrotoxicity. A potential nephrotoxicity of aminosaliclates, although rare, remains an existent possibility^[11].

Riley *et al.*^[15] showed that there was no evidence of nephrotoxicity in a cohort of patients with quiescent UC receiving mesalazine or sulfasalazine. Furthermore, Kreisel *et al.*^[17] have demonstrated that there is no positive relationship between the cumulative dose of 5-ASA and the severity of tubular microproteinuria. In accordance to these studies, we observed that tubular microproteinuria in patients with active IBD at diagnosis decreased after inducing remission of the disease, even though the patients received the treatment with 5-ASA and corticosteroids. Fraser *et al.*^[13] have demonstrated that both the prevalence and degree of tubular microproteinuria in patients with a new diagnosis of IBD are relatively unaffected by 5-ASA treatment, which is in agreement with a number of studies^[14,38,43,47]. In contrast, Schreiber *et al.*^[20] have reported that there is a positive relationship between tubular microproteinuria and cumulative 5-ASA exposure, but they questioned whether their study could provide a definite answer to the possible impact of chronic inflammation on

microproteinuria. A similar positive relationship between tubular microproteinuria and 5-ASA treatment has also been reported^[31,32].

In conclusion, abnormal mALB and tubular microproteinuria are quite frequent in IBD patients. Subclinical renal tubular and/or glomerular damage are related to the IBD itself, representing an extra-intestinal manifestation of the disease. Moreover, treatment with 5-ASA does not affect microproteinuria in IBD patients, microproteinuria in IBD patients does not predict an adverse renal response to 5-ASA.

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CLINICAL RESEARCH

Prospective, randomized, and active controlled study of the efficacy of alginic acid and antacid in the treatment of patients with endoscopy-negative reflux disease

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Abstract

AIM: To assess the efficacy and safety of a compound containing alginic acid plus antacid (Topaal[®]) compared to equal-strength antacid (Nacid[®]) in patients with endoscopy-negative reflux disease (ENRD).

METHODS: A total of 121 patients with ENRD were randomized to receive Topaal[®] (65 patients) or Nacid[®] (56 patients) for 6 weeks, with a consultation every 3 weeks. The primary end-point assessment was the change in the severity of heartburn as evaluated using a visual analog scale (VAS) at 6 weeks. The secondary end-point assessments were the VAS at 3 weeks, the change of frequency of the reflux symptom, the change of quality of life and the adverse effects.

RESULTS: Demographics of randomized subjects in each treatment group were comparable except that the Topaal[®] group included more males. The baseline characteristics between the groups were similar. After 6 weeks of treatment, the reduction of VAS of heartburn was more prominent in the Topaal[®] group (-6.29 cm vs -4.11 cm). At the 3rd week, Topaal[®] group showed greater reduction of VAS for heartburn ($P=0.0016$), regurgitation ($P=0.0006$), vomiting ($P=0.0373$), and belching ($P<0.0001$). The patients of the Topaal[®] group had lower frequency of heartburn ($P=0.0015$) and pain ($P=0.0163$) at the end of the 6-week treatment period. From the doctor's point of view, the Topaal[®] group also showed significant reduction in the severity of heartburn ($P=0.0020$), regurgitation ($P=0.0081$), vomiting ($P=0.0182$), and belching ($P=0.0018$) at the end of the treatment. The improvement of the quality of life was

more remarkable in the Topaal[®] group at the end of the 6-week treatment period ($P<0.0001$). For the adverse effect, there was no difference in both the groups.

CONCLUSION: Topaal[®] is more effective than Nacid[®] for the treatment of symptoms presented by patients with ENRD.

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Keywords: Alginic acid; Endoscopy-negative reflux disease

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INTRODUCTION

The incidence of gastroesophageal reflux disease (GERD) in Taiwan has been increasing. According to the endoscopic surveillance reports, the rate of erosive esophagitis in Taiwan has increased from 2.4% to 14.5% in the past 20 years^[1,2].

GERD is a disorder in which the gastric contents are refluxed into the esophagus, causing irritation and injury to the esophageal mucosa^[3]. The typical symptoms of GERD include heartburn and regurgitation. Less common symptoms or symptoms suggestive of more aggressive reflux disease include dysphagia, odynophagia, or gastrointestinal bleeding. Atypical complaints include chest pain, hoarseness, sore throat, chronic cough, and asthma^[3]. It has been shown that defective lower esophageal sphincter (LES) is the major mechanism underlying in most patients with reflux^[4].

Endoscopy is the most common diagnostic tool to grade and to define GERD. However, only one-third of patients with GERD have esophageal mucosal erosion or ulceration^[3]. Patients who experience typical heartburn despite that no evident mucosal lesions found at endoscopy are defined to have endoscopy-negative reflux disease (ENRD)^[5]. Compared with patients who have reflux-related erosive esophagitis, those with ENRD

are more likely to be younger, of lower body weight, and without a hiatal hernia^[6]. Approximately 50% of those with ENRD have abnormal intra-esophageal acid exposure^[6]. Although persons with ENRD experience similar decrements in their quality of life as those for individuals with erosive esophagitis, their complaints were often ignored if the diagnosis and treatment are based only on endoscopic finding. It has been proposed that the analysis of symptoms is probably the most useful method for the diagnosis of GERD^[7]. The pharmacological treatment of ENRD comprises two therapeutic classes: (1) local antacids or anti-secretory drugs including histamine receptor and proton pump inhibitors (PPI)^[8], which act by decreasing the acidity of the reflux contents; (2) alginates which act by decreasing the gastric reflux into the esophagus and by protecting the esophageal mucosa^[9]. PPI were shown to provide symptom relief in patients with ENRD^[8,10] but these treatments are not curative and not cheap. A cohort study of patients with ENRD has shown that after a median time of 10 years following the original diagnosis, 75% of patients are on prolonged antisecretory therapy because of recurrent symptoms/lesions of gastro-esophageal reflux disease^[11]. The study confirms that ENRD is a protracted disease, which requires long-term medical therapy in most of the patients. Besides, Taiwan's reimbursement system covers the PPI only when patients have endoscopic evidence of erosive esophagitis. Local antacid and alginates are more convenient and cheap options for patients with ENRD^[9]. However, there were few studies comparing the efficacy of these two classes of drug on the treatment of ENRD. We therefore carried out a prospective, randomized trial comparing the efficacy and tolerance of sodium alginate (Topaal[®]) vs antacid (Nacid[®]) in the treatment of patients with ENRD.

MATERIALS AND METHODS

Selection of patients

Disease definition: All patients who presented with the classic symptoms including heartburn and/or regurgitation, but who did not have either Barrett's esophagus or definite endoscopic esophageal mucosal breaks (esophageal mucosal erosion or ulceration) were referred to have ENRD^[7,10].

Entry procedures: The patients prior to the participation in this study signed the informed consent document, which has been approved by an ethical review board of National Taiwan University Hospital.

This study enrolled outpatients of both sexes, who were aged between 18 and 75 years, and were diagnosed to have ENRD. The included patients were asked to discontinue antacid, metoclopramide, cisapride, H₂ blocker and PPI for at least 3 days before entering the study. Those patients who had a history of intolerance or allergy to alginic acid and antacid, endoscopic evidence of esophagitis, history of partial or total gastrectomy, or had esophageal stricture, pregnancy or lactation were excluded from the enrollment.

Study endpoints

Primary efficacy endpoint: The primary efficacy endpoint was the change in the severity of heartburn as evaluated by using a visual analog scale (VAS) at the 6th

week of treatment compared to baseline in the intention-to-treat (ITT) population. The VAS^[12,13] is a 100-mm straight line with anchors (0 cm indicated no symptom and 10 cm indicated terrible) placed at both poles. Patients were asked to place a mark somewhere along the line that best described the actual status of their symptoms. The ITT population was defined as randomized patients who received at least one dose of study medication, had a baseline value and at least one post-baseline efficacy assessment. In this analysis, data from the Topaal[®] group were compared with data from Nacid[®] group.

Secondary efficacy endpoints: There were three secondary endpoints evaluated in this trial: (1) the change in the severity of reflux symptoms including heartburn, regurgitation, dysphagia, epigastric pain, nausea, vomiting and belching by VAS at the 3rd week of treatment compared to baseline; (2) the change in the frequency of heartburn, regurgitation, pain, and sleeping disturbance according to patient's diary; and (3) the change in the quality of life from doctor's point of view. All of these secondary efficacy endpoints were assessed in the ITT population.

During the 7th day before the first visit and the entire study period, each patient had to record the frequency (number of episodes per day) on the patient's diary. Each patient was asked to record the symptom of reflux disease at the moment it happened. At least a 4-d record in a week was necessary for this evaluation.

The patient's quality of life judged by the investigator's point of view was graded by modified visick grading^[11] on each visit as the following scores: no symptoms (= 1), mild symptoms easily controlled (= 2), moderate symptoms not controlled but not interfering daily life (= 3), moderate symptoms interfering daily life (= 4), and symptoms as bad or worse (= 5).

Safety endpoint: The incidence of adverse drug reaction (ADR), whether reported spontaneously, elicited by questioning, or observed by the investigators, of both Topaal[®] and Nacid[®] groups was calculated in the safety population. Safety population was defined as patients who received at least one dose of study medication after randomization. The ADR was recorded and graded according to the WHO definition^[14]. The investigators also assessed the causal relationship of any adverse event to the study medication by using the ADR probability scale which was generated from Naranjo *et al*^[15].

All routine laboratory variables (hematology and serum chemistry) were recorded at each visit. Every assessment was compared with baseline values.

Allocation of patients

This was a prospective, randomized, open-label and active-controlled study. Doctors prescribed the test drugs according to the random number sheet, which is generated by SAS. Eligible patients were allocated the next sequential patient number based on preprinted numbers on the study drug labels.

Patients with symptomatic GERD will undergo endoscopy. Randomization occurred within 7 days of the baseline endoscopy. Patients with ENRD were enrolled into the study and then randomly allocated to treat with

Table 1 Demographics between groups- (safety populations)

| | All (134) | Topaal (69) | Nacid (65) | P |
|---|--------------|----------------|---------------|---------------------|
| Sex | | | | |
| Male:Female | 36:98 | 25:44 | 11:54 | 0.0186 ¹ |
| Age (yr) | 41.9 ± 13.4 | 41.6 ± 14.8 | 42.4 ± 11.8 | 0.7300 ² |
| Body mass index (kg/m ²) | 23.1 ± 3.8 | 23.4 ± 3.8 | 22.8 ± 3.8 | 0.3547 ² |
| Pulse rate (bpm) | 75.2 ± 6.8 | 75.3 ± 7.0 | 75.1 ± 6.7 | 0.9075 ² |
| SBP (mmHg) | 117.0 ± 14.1 | 119.7 ± 14.2 | 114.1 ± 13.5 | 0.0211 ² |
| DBP (mmHg) | 75.0 ± 1.01 | 76.6 ± 9.5 | 73.2 ± 10.6 | 0.0516 ² |
| History of heartburn (%) | | | | 0.9658 ³ |
| 6-12 mo | 75 (56.4) | 39 (56.5) | 36 (56.3) | |
| 1-5 yr | 45 (33.8) | 23 (33.3) | 22 (34.4) | |
| >Section 15 yr | 13 (9.8) | 7 (10.1) | 6 (9.4) | 0.4011 ⁴ |
| Smoking (%) | | | | |
| Yes | 14 (10.5) | 9 (13.0) | 5 (7.7) | |
| No | 120 (89.5) | 60 (87.0) | 60 (92.3) | 1.0000 ⁴ |
| Drinking (%) | | | | |
| Yes | 5 (3.7) | 3 (4.4) | 2 (3.1) | |
| No | 129 (96.3) | 66 (95.6) | 63 (96.9) | 0.0881 ⁴ |
| Coffee (%) | | | | |
| Yes | 28 (20.9) | 10 (14.5) | 18 (27.7) | |
| No | 106 (79.1) | 59 (85.5) | 47 (72.3) | |
| VAS (cm) | | | | 0.8388 ² |
| Heartburn | 7.5 ± 3.4 | 7.6 ± 3.0 | 7.4 ± 3.7 | |
| Regurgitation | 8.2 ± 3.0 | 8.1 ± 3.1 | 8.2 ± 2.9 | |
| Dysphagia | 1.7 ± 3.2 | 1.4 ± 3.0 | 2.0 ± 3.5 | |
| Epigastric pain | 5.4 ± 4.1 | 4.6 ± 4.1 | 6.2 ± 3.9 | |
| Nausea | 3.5 ± 3.8 | 3.3 ± 3.8 | 3.7 ± 3.8 | |
| Vomiting | 1.1 ± 2.7 | 1.2 ± 2.7 | 1.0 ± 2.6 | |
| Belching | 6.2 ± 4.0 | 5.9 ± 3.9 | 6.4 ± 4.1 | |
| Quality of life (%) | | | | 0.8489 ³ |
| Mild symptoms | 4 (3.0) | 3 (4.4) | 1 (1.5) | |
| Moderate w/o interfering | 33 (24.6) | 15 (21.7) | 18 (27.7) | |
| Moderate with interfering | 66 (49.3) | 36 (52.2) | 30 (46.2) | |
| Symptom as bad/worse | 31 (23.1) | 15 (21.7) | 16 (24.6) | |

¹Fisher's exact test; ²ANOVA model; ³Mantel-Henszel test; VAS: visual analog scale.

either Topaal[®] or Nacid[®]. No other medical treatment for reflux esophagitis such as other antacid, metoclopramide, cisapride, H₂ blocker and PPI was allowed.

The duration of treatment was 6 wk and each patient returned for assessment every 3 wk. At randomization (visit 1) and visit 2, all patients will received one plastic bottle of study medication. Every bottle contains 200 chewable tablets of Topaal[®] or Nacid[®], including 4-day supply in the event of a delay in the scheduled appointment. The dosage of Topaal[®] and Nacid[®] is two chewable tablets t.i.d. and h.s.

At the initial assessment, the medical history, past history, life-style (smoking, drinking, coffee consumption) and baseline demographic data, hematologic and serum biochemistry were recorded. A physical examination will be by performed.

Treatment medications

Drug information: Topaal[®] contains 200 mg alginic acid, 30 mg colloidal aluminum hydroxide and 40 mg magnesium hydrocarbonate per tablet. Nacid[®] contains 500 mg Mg₆Al₂(OH)₁₆CO₃·4H₂O per tablet.

Topaal[®] is an alginate-based raft-forming formulation^[16]

Table 2 The change in the VAS of heartburn (ITT population)

| Unit: cm | Topaal Mean (SE) | Nacid Mean (SE) | Difference | P |
|----------------------|---------------------|--------------------|------------|---------|
| Visit 1/baseline | | | | |
| Observed data | 7.52 (0.42) | 7.43 (0.45) | 0.09 | 0.8774 |
| Visit 3/week 7 | | | | |
| Observed data | 1.15 (0.30) | 3.06 (0.34) | -1.91 | <0.0001 |
| Change from baseline | -6.39 (0.28) | -4.44 (0.31) | -1.95 | <0.0001 |

ANCOVA model: Dependent variable = (baseline value) + treatment.

comprising alginic acid and antacid. In the presence of gastric acid, alginates precipitate and form a gel^[16]. The bicarbonate inside is converted to carbon dioxide, which is then entrapped within the gel precipitate, converting it into foams floating on the surface of the gastric contents, much like a raft on water. The "raft" can act as a physical barrier to reduce reflux episodes.

Statistical analysis

Comparisons of frequency employed the χ^2 test of Fisher's exact test, the test of linear trend for the ordered variables, Student's *t*-test of non-parametric Wilcoxon test, and variance analysis for the repeated VAS, all adjusted to the initial values. Two-sample *t* test was used to compare the continuous data such as height, weight, age, the number of heartburn, the number of sleeping disturbance etc., and the 95%CI for the difference were calculated. Statistical significance was assessed at the 5% level. The principle analysis was carried out on an ITT basis.

A listing of patients with withdrawal as well as those with premature termination of study drugs, along with the date and reasons for the termination was provided.

RESULTS

Disposition of patients

The trial was conducted from June 10, 2003 (first patient screened) to December 24, 2004 (last patient completed). Figure 1 gives an overview of the disposition of the patient population for the treatment phases. A total of 134 patients were randomized to the treatment phase; 69 patients were randomized to Topaal[®] and 65 were randomized to Nacid[®]. Of the 134 randomized patients, a total of 112 patients completed the study.

The safety population included 134 patients (69 in the Topaal[®] group, 65 in the Nacid[®] group) who received at least one dose of study medication after randomization. The ITT population consisted of all randomized subjects who received at least one dose of study treatment and who had a baseline and at least one post-baseline efficacy assessment. Three of the sixty-five patients in Nacid[®] group dropped out for safety/efficacy reason; six in Nacid[®] group and four in the Topaal[®] group dropped out for administrative reasons (e.g., lost to follow-up, moved out of the area, etc.). Therefore, 65 patients in the Topaal[®] group and 56 patients in the Nacid[®] group were included in the efficacy population.

Table 3 Summary of the changes in the VAS at 3 weeks (ITT population)

| Unit: cm | Topaal (65) | | Nacid (56) | | Difference | P-value |
|----------------------|-------------|------|------------|------|------------|---------|
| | Mean | (SE) | Mean | (SE) | | |
| Heartburn | | | | | | |
| Observed data | 2.98 | 0.37 | 4.43 | 0.40 | -1.44 | 0.0091 |
| Change from baseline | -4.52 | 0.31 | -3.03 | 0.34 | -1.49 | 0.0016 |
| Regurgitation | | | | | | |
| Observed data | 3.52 | 0.37 | 5.32 | 0.40 | -1.80 | 0.0012 |
| Change from baseline | -4.55 | 0.33 | -2.84 | 0.36 | -1.72 | 0.0006 |
| Dysphagia | | | | | | |
| Observed data | 0.65 | 0.27 | 1.25 | 0.29 | -0.60 | 0.1326 |
| Change from baseline | -0.97 | 0.19 | -0.68 | 0.20 | -0.28 | 0.3138 |
| Epigastric pain | | | | | | |
| Observed data | 2.08 | 0.42 | 3.88 | 0.45 | -1.80 | 0.0039 |
| Change from baseline | -2.93 | 0.33 | -2.10 | 0.36 | -0.84 | 0.0925 |
| Nausea | | | | | | |
| Observed data | 1.25 | 0.32 | 1.89 | 0.34 | -0.65 | 0.1705 |
| Change from baseline | -2.10 | 0.25 | -1.51 | 0.27 | -0.59 | 0.1137 |
| Vomiting | | | | | | |
| Observed data | 0.31 | 0.18 | 0.61 | 0.20 | -0.30 | 0.2662 |
| Change from baseline | -0.92 | 0.12 | -0.55 | 0.13 | -0.37 | 0.0373 |
| Belching | | | | | | |
| Observed data | 2.54 | 0.40 | 5.14 | 0.43 | -2.60 | <0.0001 |
| Change from baseline | -3.64 | 0.32 | -1.40 | 0.34 | -2.23 | <0.0001 |

ANCOVA model: Dependent variable = (baseline value) + treatment.

Demographics and baseline characteristics

Demographic characteristics of randomized subjects in each treatment group are summarized in Table 1. The mean age of the patients in both treatment groups was about 42 years. There were more female than male patients participating in the study in both groups. Although there were significant differences in gender ratio between groups (36.23% male in the Topaal[®] group *vs* 16.92% male in the Nacid[®] group), no evidence showed that the gender difference would have an effect on the pharmacokinetics or efficacy of study medications. The body mass index (BMI), pulse rate and diastolic blood pressure were all comparable between treatment groups. Although the patients in the Topaal[®] group seem to have higher systolic blood pressure than the patients in the Nacid[®] group (119.7 ± 14.2 mmHg in the Topaal[®] group *vs* 114.1 ± 13.5 mmHg in the Nacid[®] group), those values were all clinically considered to be within normal limits.

Table 1 also summarizes patients' baseline symptom analysis that included VAS, the reflux symptoms from doctor's point of view, and quality of life from doctor's point of view. More than 56% of the patients in each group had suffered from the reflux symptom of heartburn for at least 6-12 mo. No significant difference in other characteristics including life-style was noted. The baseline characteristics of all randomized subjects were compatible between the two treatment groups, and no statistically significant difference between treatment groups was detected, except for the mean VAS of epigastric pain ($P = 0.0218$).

EFFICACY EVALUATION

The change in the severity of the heartburn as evaluated using a VAS at 6 wk, using LOCF data from ITT population, was the primary efficacy endpoint of this trial. At

baseline, the mean VAS of heartburn was 7.52 cm in the Topaal[®] group and 7.43 cm in the Nacid[®] group. At the end of the 6-week treatment period, the mean VAS of heartburn was 1.20 cm in the Topaal[®] group and 3.36 cm in the Nacid[®] group. Overall, the mean change from baseline in VAS of heartburn over the 6-weeks treatment was -6.29 cm (-6.85 to -5.74 cm) in the Topaal[®] group, and -4.11 cm in the Nacid[®] group (-4.71 to -3.51 cm). The mean difference between the two treatment groups was -2.19 cm (-3.01 to -1.37 cm) and the difference was statistically significant ($P < 0.0001$) as detailed in Table 2.

The first secondary endpoint was to compare the change of VAS at 3 wk (Table 3). There were significant differences in the mean change of VAS for heartburn ($P = 0.0016$), regurgitation ($P = 0.0006$), vomiting ($P = 0.0373$) and belching ($P < 0.0001$). No significant differences were found in the mean change of VAS for nausea, epigastric pain, and dysphagia. The results suggested that Topaal[®] is superior to Nacid[®] for the improvement of the predominant symptoms of ENRD such as heartburn and regurgitation.

The second secondary endpoint was to compare the frequency of heartburn, regurgitation, pain, and sleep disturbance according to patient's diary every week during treatment period (Figure 2). Patients taking Topaal[®] showed consistently and significantly lower frequency of heartburn than those who were taking Nacid[®] throughout the entire 6-week period ($P = 0.0015$). Patients taking Topaal[®] also had fewer episodes of pain than those who were taking Nacid[®] from the 4th week to the end of the study. Both Topaal[®] and Nacid[®] decreased the frequency of regurgitation during the study period compared to baseline, but the difference between treatments was not significant. No difference was observed for the change in the frequency of sleeping disturbance.

The third and fourth secondary endpoints were to

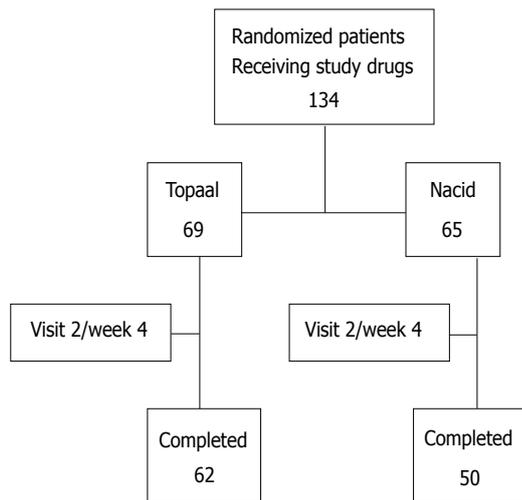


Figure 1 Disposition of patients in the treatment phase.

compare the change in the severity of the reflux symptoms (Table 4) and the quality of life from doctor's point of view (Table 5). There were no significant differences in the severity of the reflux symptoms and the quality of life from doctor's point of view at baseline. At the end of the 6-week treatment period, patients who took Topaal[®] showed better improvement in the severity of heartburn ($P=0.0020$), regurgitation ($P=0.0081$), vomiting ($P=0.0182$), and belching ($P=0.0018$) than those who took Nacid[®]. There were no significant differences in the improvement of dysphagia ($P=0.7551$), epigastric pain ($P=0.2648$), and nausea ($P=0.0577$).

For the quality of life from doctor's point of view, there were no significant differences at baseline. About 50% of the patients in each group suffered from moderate reflux symptoms interfering with the quality of life before the treatment. At the end of treatment period, patients who took Topaal[®] had greater alleviations of symptoms ($P<0.0001$) and greater reduction of symptom score from baseline ($P<0.0001$) than those who took Nacid[®]. The results suggested that Topaal is more efficient than Nacid for improving the quality of life of ENRD patients.

SAFETY EVALUATION

The ADRs of Topaal[®] and Nacid[®] were the safety endpoints of this trial. In this analysis, data from the Topaal[®] group were compared with data from Nacid[®] group.

Adverse events which occurred during the course of the study were recorded. Seven patients had at least one adverse event after entering the study, three (4.35%) patients in the Topaal[®] group and four (6.15%) patients in the Nacid[®] group. The most commonly adverse events reported by Topaal[®]-treated patients were constipation (2 patients, 2.90%). For Nacid[®] group, two patients (3.08%) experienced diarrhea and two patients (3.08%) reported constipation. No statistical significant difference was found between the two groups in the incidence of adverse event. All of these adverse events were resolved and graded as mild or moderate in severity. None of them was considered by the investigators to be definitely related to treatment. No dosage modification of medication was

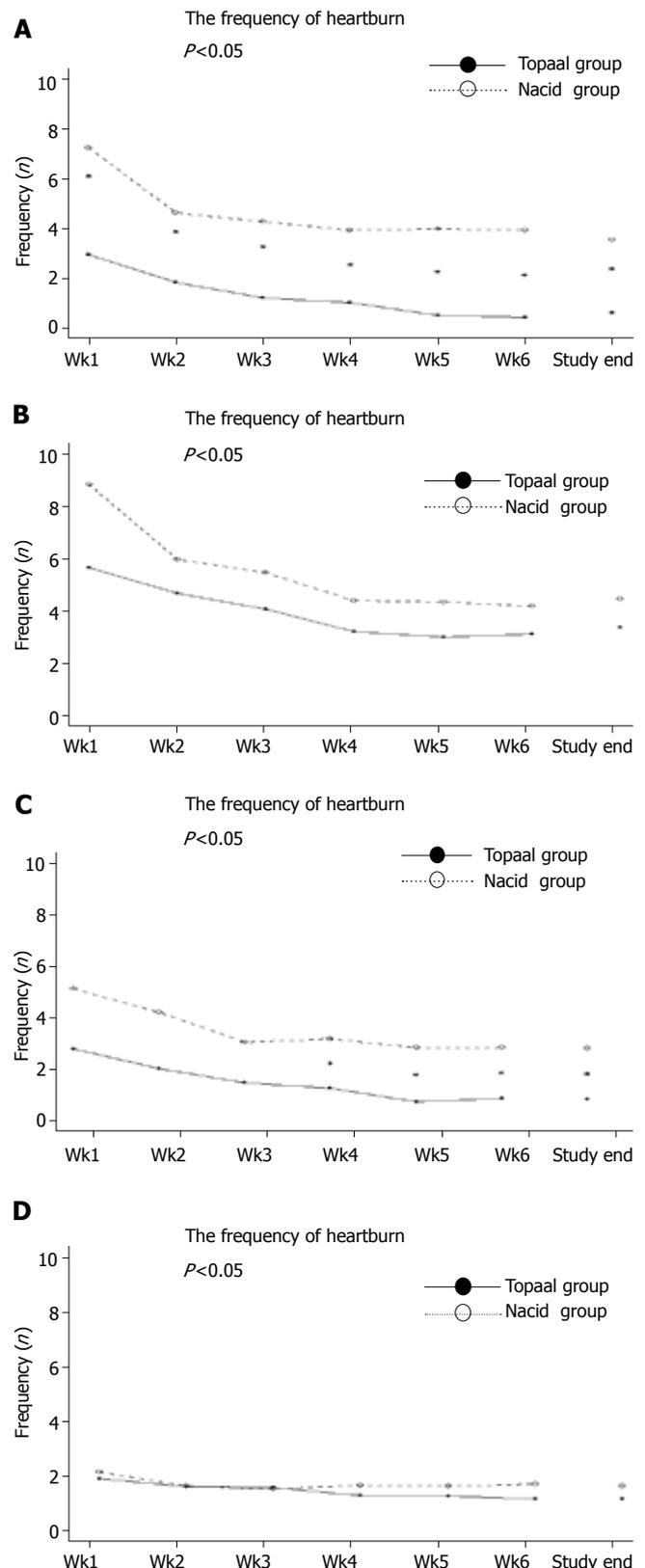


Figure 2 The frequency of heartburn, regurgitation, pain and sleep disturbance.

applied for the three adverse events in Topaal[®]-treated patients, whereas two of the four adverse events in Nacid[®]-treated patients led to dose reduction of medication. No serious adverse event was reported during the entire study period. The results suggest that Topaal[®] had a similar safety profile as Nacid[®].

Table 4 The change in the severity of the reflux symptom

| Population: intent-to-treat | Overall (%) | Topaal (%) | Nacid (%) | P-value |
|-----------------------------|-------------|------------|-----------|---------------------|
| Heartburn | | | | 0.0019 ¹ |
| Change from baseline | | | | |
| -1 | 4 (3.3) | 1 (1.5) | 3 (5.4) | |
| 0 | 28 (23.1) | 8 (12.3) | 20 (35.7) | |
| 1 | 42 (34.7) | 21 (32.3) | 21 (37.5) | |
| 2 | 30 (24.8) | 23 (35.4) | 7 (12.5) | |
| 3 | 17 (14.1) | 12 (18.5) | 5 (8.9) | |
| Regurgitation | | | | 0.0625 ¹ |
| Change from baseline | | | | |
| -1 | 2 (1.6) | 1 (1.5) | 1 (1.8) | |
| 0 | 30 (24.8) | 12 (18.5) | 18 (32.1) | |
| 1 | 42 (34.7) | 21 (32.3) | 21 (37.5) | |
| 2 | 36 (29.8) | 21 (32.3) | 15 (26.8) | |
| 3 | 11 (9.1) | 10 (15.4) | 1 (1.8) | |
| Dysphagia | | | | 0.2559 ¹ |
| Change from baseline | | | | |
| -1 | 4 (3.3) | 2 (3.1) | 2 (3.8) | |
| 0 | 90 (74.4) | 50 (79.6) | 40 (71.4) | |
| 1 | 16 (13.2) | 5 (7.5) | 11 (19.6) | |
| 2 | 10 (8.3) | 7 (10.8) | 3 (5.3) | |
| 3 | 1 (0.8) | 1 (1.5) | 0 (0.0) | |
| Epigastric pain | | | | 0.2517 ¹ |
| Change from baseline | | | | |
| -2 | 3 (2.5) | 1 (1.5) | 2 (3.8) | |
| -1 | 4 (3.3) | 3 (4.6) | 1 (1.8) | |
| 0 | 57 (47.1) | 30 (46.2) | 27 (48.2) | |
| 1 | 33 (27.3) | 15 (23.1) | 18 (32.1) | |
| 2 | 19 (15.7) | 11 (16.9) | 8 (14.3) | |
| 3 | 5 (4.1) | 5 (7.7) | 0 (0.0) | |
| Nausea | | | | 0.2210 ¹ |
| Change from baseline | | | | |
| -1 | 1 (0.8) | 0 (0.00%) | 1 (1.8) | |
| 0 | 70 (57.9) | 33 (50.8) | 37 (66.1) | |
| 1 | 23 (19.0) | 15 (23.1) | 8 (14.3) | |
| 2 | 25 (20.7) | 15 (23.1) | 10 (17.9) | |
| 3 | 2 (1.7) | 2 (3.1) | 0 (0.0) | |
| Vomiting | | | | 0.1888 ¹ |
| Change from baseline | | | | |
| -1 | 1 (0.8) | 0 (0.0) | 1 (1.8) | |
| 0 | 100 (82.6) | 50 (79.6) | 50 (81.3) | |
| 1 | 9 (7.4) | 6 (9.2) | 3 (5.4) | |
| 2 | 9 (7.4) | 7 (10.8) | 2 (3.6) | |
| 3 | 2 (1.7) | 2 (3.1) | 0 (0.0) | |
| Belching | | | | 0.0366 ¹ |
| Change from baseline | | | | |
| -1 | 8 (6.6) | 2 (3.1) | 6 (10.7) | |
| 0 | 48 (39.7) | 21 (32.3) | 27 (48.2) | |
| 1 | 42 (34.7) | 25 (38.5) | 17 (30.4) | |
| 2 | 19 (15.7) | 13 (20.0) | 6 (10.7) | |
| 3 | 4 (3.3) | 4 (6.2) | 0 (0.0) | |

χ^2 test.

DISCUSSION

The design of this study is based on symptomatic analysis. This is an original, though not blind, trial that compares the therapeutic effects of an alginate to an antacid on ENRD patients. For the best interest of the patients, a placebo-controlled study was not considered in our design. The investigators' and the patients' assessments of efficacy over the study period are similar and in favor of the alginate. After 6-week treatment, Topaal[®] was found to be more effective than Nacid[®] in reducing VAS of five symptoms including heartburn, regurgitation, vomiting,

and belching in patients with ERND. Patients in Topaal[®] group had fewer episodes of heartburn and epigastric pain at the end of treatment. Also, from doctor's point of view, Topaal[®] is more effective than Nacid[®] in reducing symptoms of reflux and in improving the quality of life in patients with ERND. Our result is consistent with that of other trials that compared the effects of alginate-based formulation with antacids on patients with reflux esophagitis^[17] and volunteers^[18], though their patient groups were more heterogeneous.

According to our data, the symptom-relieving effect in the Topaal[®] group was faster than that of Nacid[®]. Patients

Table 5 Summary of the change of quality of life

| ITT population | Overall (%) | Topaal (%) | Nacid (%) | P-value |
|-----------------------------------|-------------|------------|-----------|---------|
| Visit 1/baseline | | | | |
| Observed data | | | | 0.9298 |
| No symptom | 0 (0.0) | 0 (0.0) | 0 (0.0) | |
| Moderate symptom | 4 (3.3) | 3 (4.6) | 1 (1.8) | |
| Moderate symptom w/o interfering | 29 (24.0) | 13 (20.0) | 16 (28.6) | |
| Moderate symptom with interfering | 61 (50.4) | 35 (53.9) | 26 (46.4) | |
| Symptom as bad/worse | 27 (22.3) | 14 (21.5) | 13 (23.2) | |
| Study end | | | | |
| Observed data | | | | <0.0001 |
| No symptom | 24 (19.8) | 19 (29.2) | 5 (8.9) | |
| Moderate symptom | 51 (42.2) | 34 (52.3) | 17 (30.4) | |
| Moderate symptom w/o interfering | 27 (22.3) | 8 (12.3) | 19 (33.9) | |
| Moderate symptom with interfering | 15 (12.4) | 3 (4.6) | 12 (21.4) | |
| Symptom as bad/worse | 4 (3.3) | 1 (1.5) | 3 (5.4) | |
| Decrease from baseline | | | | <0.0001 |
| -1 | 1 (0.8) | 0 (0.0) | 1 (1.8) | |
| 0 | 24 (19.8) | 7 (10.8) | 17 (30.4) | |
| 1 | 36 (29.8) | 16 (24.6) | 20 (35.7) | |
| 2 | 36 (29.8) | 22 (33.9) | 14 (25.0) | |
| 3 | 16 (13.2) | 13 (20.0) | 3 (5.4) | |
| 4 | 8 (6.6) | 7 (10.8) | 1 (1.8) | |

¹Mantel-Henszel χ^2 test.

taking Topaal[®] had major reduction in VAS scores of heartburn and regurgitation from the 1st week of study, and patients taking Nacid[®] did not feel the difference in symptoms until the 2nd week of study. Alginic acid is different from antacids in the mechanism of efficacy. The alginic acid is converted to sodium alginate by the small amount of antacid contained in the formulation. This salt floats atop of the esophagogastric junction and provides a barrier when reflux occurs^[16]. The physical property of Topaal[®] might explain why it worked faster and better than Nacid[®] in relieving the symptoms of reflux. On the other hand, our results did not show differences in improving the sleep disturbance in patients of both groups. We did not assess the incidence of night refluxer or asthma in the enrolled patients who might have greater disturbance in sleeping. We deduced that the incidence of night refluxer maybe low because both study groups had low VAS score of sleep disturbance at the baseline. Though alginate products have been shown to be effective in relieving symptoms of reflux, it has not been proved that the alginates promote healing of erosive esophagitis. In this way, alginate-based formulation is a reasonable option for patients with ENRD.

Goves *et al*^[19] compared the effectiveness of PPI, with alginates for heartburn relief in dyspeptic patients. This design showed that PPI was superior to alginates in relieving the heartburn of patients at 4 weeks of treatment. This is not surprising for they included patients with severe esophagitis, and omeprazole had the dramatic antisecretory actions. However, considering ENRD is a protracted disease, alginate is a better option for long-term and on-demand use in these patients for cost-effectiveness. For adverse events, Topaal[®] group had a compatible safety profile as Nacid[®] group. Alginates have been shown to be safe when used in pregnant women^[20] and children^[21]. In our patients treated with Topaal[®], the most commonly

reported adverse events were constipation (2.90%).

In conclusion, Topaal[®] is more effective than Nacid[®] in symptomatic control of patient with ENRD. For the safety measures, the results supported that Topaal[®] group had a compatible safety profile as Nacid[®] group.

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Clinical application of self-expanding metallic stent in the management of acute left-sided colorectal malignant obstruction

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Abstract

AIM: To summarize our experience with the application of self-expanding metallic stent (SEMS) in the management of acute left-sided colorectal malignant obstruction.

METHODS: A retrospective chart review of all patients undergoing placement of SEMS between April 2000 and January 2004 was performed.

RESULTS: Insertion of SEMS was attempted in 26 patients under fluoroscopic guidance with occasional endoscopic assistance. The sites of lesions were located in splenic flexure of two patients, left colon of seven patients, sigmoid colon of eight patients and rectum of nine patients. The intended uses of SEMS were for palliation in 7 patients and as a bridge to elective surgery in 19 patients. In the latter group, placement of SEMS allowed for preoperative systemic and bowel preparation and the following one-stage anastomosis. Successful stent placement was achieved in 22 (85%) of the 26 patients. The clinical bowel obstruction resolved 24 hours after successful stent placement in 21 (95%) patients. Three SEMS-related minor complications occurred, two stents migrated and one caused anal pain.

CONCLUSION: SEMS represents an effective and safe tool in the management of acute malignant colorectal obstruction. As a bridge to surgery, SEMS can provide time for systematic support and bowel preparation and obviate the need for fecal diversion or on-table lavage. As a palliative measure, SEMS can eliminate the need for emergent colostomy.

INTRODUCTION

Acute left-sided colorectal malignant obstruction represents a frequent surgical emergency. It is the first symptom in approximately 15-20% of colorectal cancers^[1-2]. Single-stage surgery (subtotal colectomy with primary anastomosis or partial colectomy with intraoperative lavage) appears to be gaining acceptance as a good therapeutic option in such cases. However, this treatment option is not feasible in all patients, and it is often necessary to use a two-stage procedure (Hartmann's procedure) or to perform permanent colostomy in advanced stages of the disease^[3]. Smothers *et al.*^[4] found that the overall surgical morbidity and mortality are significantly higher in patients with colon cancer undergoing emergency surgery than in those undergoing selective surgery. Some other factors such as advanced age as well as stage and volume of the disease are closely correlated with emergency surgery, and emergency surgery has a strong independent negative influence on immediate surgical morbidity and mortality. If emergency surgery could be transferred to elective surgery after a simple effective therapy, complications would be significantly decreased in patients with acute left-sided colorectal malignant obstruction.

SEMS^[5-7] has been used to relieve obstruction in different situations. Application of metallic stents in the treatment of acute malignant colonic obstruction was first reported by Dohmoto^[8] in 1991 and has become a promising treatment option, though the number of reported cases is only about 600 in the world^[9,10]. Placement of SEMS across the obstructing lesion can relieve obstructive symptoms and avoid emergency surgery. In most cases, a radical colectomy with primary



Figure 1 Endoscopic view of partially expanded stent immediately after the stent placement.

anastomosis can be subsequently performed in 6-9 days. Colostomy is the only surgical option, and implantation of stent is the best final palliative treatment in some cases with advanced stages of the disease^[11-23]. This report describes the experience with the application of SEMS in the management of acute malignant left-sided colorectal obstruction.

MATERIALS AND METHODS

Patients

All patients had clinical symptoms and signs of acute bowel obstruction. From April 2000 to January 2004, a total of 26 patients (16 men, 10 women; mean age: 63.23 years; range: 38-85 years) were selected to receive decompressive therapy by implanting SEMS. Patient selection criteria included the site of obstruction from the middle rectum to the descending colon and absence of bowel necrosis and perforation. Patients with more proximal obstructions were excluded on the basis of poor site accessibility and low emergency surgery risk. Age, general condition, and tumor stage were not used as exclusion criteria.

All patients received plain radiography of the abdomen and emergency contrast-enhanced computed tomography. The sites of obstruction were located in rectum of nine patients, sigmoid colon of eight patients, descending colon of seven patients and splenic flexure of two patients. Informed consent was obtained from all patients or from their relatives after the risks and benefits of treatment were fully explained.

Procedure

All procedures were performed at the interventional radiology by endoscopy (Figure 1) and fluoroscopy (Figure 2). Neither analgesia nor sedation was administered during the procedure. There was no routine administration of antibiotics.

Once the guide wire passed through the lesion, the catheter was advanced to the most proximal point, and contrast medium was injected to evaluate tumor length and exclude the possibility of perforation. The superstiff 0.038-inch guide wire was then inserted, and a stent was placed using the delivery system under fluoroscopic control (Figure 2).

Several silver clips were clipped around the lesion during colonoscopy. Then the guide wire inserted through the lesion under direct vision was confirmed to arrive at the splenic flexure under fluoroscopic monitor.

The stent used in all patients was esophageal endoprosthesis (Nanjing Microinvasive Corporation, China), 20 or 25 mm in diameter and 70-100 mm in length. After the placement of the stent, a catheter was inserted over the superstiff guide wire until the tip projected a few centimeters beyond the stent. Contrast medium was injected to ensure that the stent was placed properly across the tumor and to assess the stent patency.

When one stent was not long enough to span the lesion, a second stent was inserted to overlap the first. Balloon dilation was not performed in any of the patients either before or after the implantation of the stent.

Patients were transferred to the surgical ward for observation after the procedure. Abdominal plain radiography was performed to assess the stent expansion and patency as well as possible complications in 24 h. Once symptoms of obstruction remitted, further tumor staging and clinical evaluation were carried out to determine which patients should receive selective surgical therapy and which patients should undergo stent implantation as the definitive palliative treatment.

RESULTS

Successful stent placement was achieved in 22 (85%) of the 26 patients. The procedure could not be completed in four patients. The sites of lesions were located in splenic flexure of one patient, descending colon of two patients and sigmoid colon of one patient. The successful rate was 50% in splenic flexure (1/2), 71% in the descending colon (5/7), 89% in sigmoid colon (7/8), 100% in rectum (9/9), respectively. The obstruction could not be relieved in three patients even though the guide wire and catheter were advanced successfully to the edge of the lesion. Though the guide wire was successfully advanced proximally, the stent could not be implanted in the remaining patient because of the tortuosity of the rectosigmoid region. Emergency laparotomy was performed in these four patients, and colostomy or Hartmann's procedure was completed.

A total of 25 self-expanding metallic stents were implanted. One stent was placed in 19 patients and two stents in 3 patients. The mean fluoroscopy time of the procedure was 46 min (range: 15-120 min).

The clinical bowel obstruction resolved in 21 patients (21/22, 95%) within 24 h after successful stent placement, with expansion and patency observable in all radiographic films. Clinical failure occurred in one patient and symptom of incomplete obstruction persisted for 24 h following the successful placement of SEMS. Emergency laparotomy revealed vegetable residues proximal to the lesion and Hartmann's procedure was performed. Complications of SEMS placement occurred in three patients. Stent migration occurred in two patients after obstruction disappeared but no re-obstruction was found after stent migration. Mild anal pain persisted for one month in the remaining one because the stent was close to the anus. The

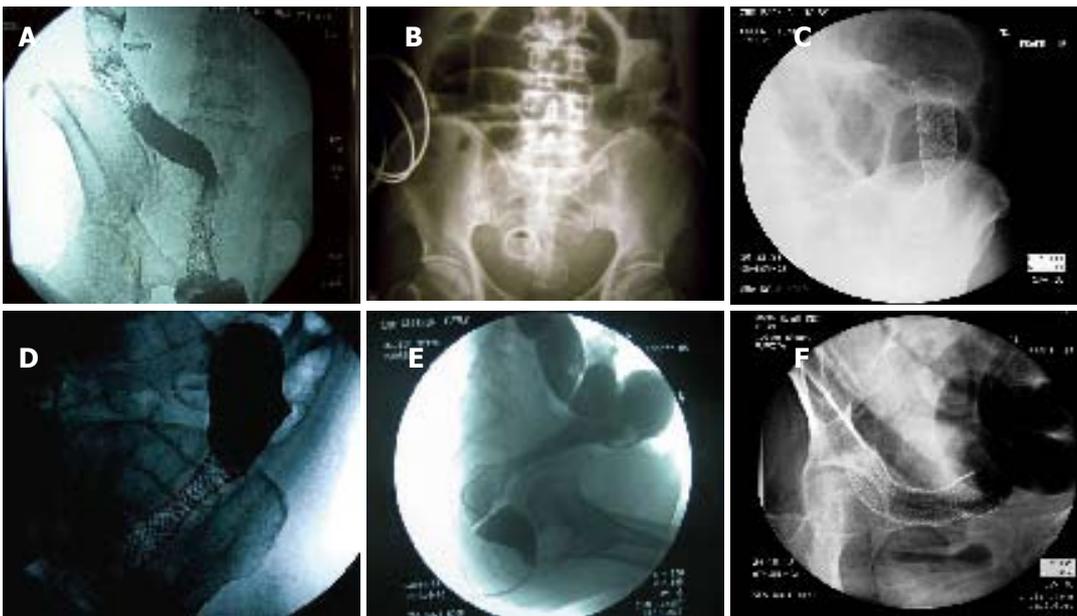


Figure 2 Good patency of the second stent coaxially placed covering the entire malignant stricture (A), a patent and nearly full in the upper rectum (B), SEMS with a waist shape in descending colon cancer (C), SEMS bridging the descending colon (D), a patent stent after SEMS placement in the rectum (E) and a bent stent in sigmoid cancer (F).

pain resolved after analgesic was administered (Table 1).

Surgery

Patients with operable colorectal carcinoma were considered as candidates for selective surgery. The mean time between stent placement and surgery was 8 ± 3 days (range: 5-11 days). These patients underwent adequate bowel preparation and adjuvant systematic therapy. The stent did not interfere with or prolong the surgery in any of these patients. The stents were fully expanded as well as in good position and afforded sufficient tumoral coverage (except for two patients with stent migration) and adequate cleaning of the colon in all patients during surgical exploration. Left colectomy, sigmoidectomy, and (low) anterior resection were performed in five patients, respectively.

Palliation

Stent placement was considered as the definitive palliative treatment of colonic obstruction in seven patients. Lesions were located at the sigmoid of three patients and rectum of four patients. Two patients suffered from gastric carcinoma with sigmoid or rectal seeding and three patients had primary sigmoid or rectal carcinomas with inoperable liver metastases. One patient with sigmoid carcinoma failed in stenting and underwent Hartmann's procedure. The other six patients received adjuvant chemotherapy, or radiation therapy, or both.

DISCUSSION

Usually, it is not difficult to detect acute left-sided colorectal obstruction by abdominal radiography. CT can help find the site and etiology of colon stenosis and the nature of acute colonic occlusion. Barium enema for the diagnosis and treatment of colonic obstruction is not recommended when doctors plan to implant SEMS^[12].

SEMS for colorectal obstruction was performed in the past either under radiological or endoscopic control. We introduced the guide wire under radiological and

endoscopic guidance in most patients. The guide wire could be inserted under direct vision, radiation exposure could be reduced, and biopsies for histology could be taken during the procedure.

Endoscopic placement of SEMS is advantageous over radiological placement because the accessibility to colorectal tumor sites is greater and stents can directly pass through the working channel of the endoscope^[13]. These advantages are especially obvious when the obstruction is proximal to the rectosigmoid. De Gregorio *et al.*^[14] have reported that the guide wire cannot be progressed under fluoroscopic guidance but reaches the neoplastic stricture under endoscopic guidance. Interventional radiologists are much more experienced in passing the guide wires through obstructive lesions and deploying SEMS^[12,13]. In our study, fluoroscopy in combination with endoscopy improved SEMS deployment. Even then, stent was still rather difficult to implant in the descending colon and splenic flexure compared with that in the rectum and sigmoid (6/9, 67%; 16/17, 94%; $P < 0.05$).

We treated 26 patients with acute malignant colorectal obstruction with SEMS placement with a successful rate of 85% (22/26). Only one patient (5%, 1/22) failed to relieve obstruction due to vegetable residues. If we had used colonoscopy to detect and get out the vegetable residues, the patient could have the obstruction relieved and emergency colostomy could not have been performed (Hartmann's procedure). After successful stent placement and proper bowel preparation, 15 patients achieved elective radical colorectal surgery and anastomosis without severe complication. Six patients with inoperable malignancy obviated emergency colostomy after successful stent placement. Although 85% of successful stent placement was a little lower than 90-95% of some foreign reports, we believe that success rate of SEMS placement would be further increased with more refined Chinese instruments and improved manipulation skill.

It was reported that SEMS placement can be used as a palliative treatment for colonic obstruction. In Cristina's study, in 17 (of 43) patients with Dukes D tumor (40%),

Table 1 Details of patients with colorectal obstruction after SEMS placement

| Patient No. (gender/age yr) | Diagnosis | Site of obstruction | Nature of treatment | Stent insertion | Stent number | Subsequent treatment | Complication |
|-----------------------------|--|-------------------------|---------------------|-----------------|--------------|----------------------|--------------------------|
| 1 (F/45) | Primary Ca descending | Splenic flexure | Temporizing | Failed | - | Colostomy | No |
| 2 (F/48) | Primary Ca rectum, ascites, liver metastasis | Middle rectum | Palliative | Successful | 2 | Chemo-therapy | No |
| 3 (F/73) | Primary Ca rectum | Middle rectum | Temporizing | Successful | 1 | LAR | No |
| 4 (F/38) | Primary Ca stomach | Sigmoid | Palliative | Successful | 2 | Chemo-radio therapy | No |
| 5 (M/61) | Primary Ca descending | Descending upper | Temporizing | Successful | 2 | Left colectomy | No |
| 6 (M/83) | Primary Ca rectum | Rectum | Temporizing | Successful | 1 | AR | No |
| 7 (M/72) | Primary Ca descending | Descending | Temporizing | Failed | - | Colostomy | No |
| 8 (M/76) | Primary Ca sigmoid, liver metastasis | Sigmoid | Palliative | Successful | 1 | Chemo-therapy | No |
| 9 (F/44) | Primary Ca rectum | Upper and middle rectum | Temporizing | Successful | 1 | LAR | No |
| 10 (F/74) | Primary Ca sigmoid | Sigmoid | Palliative | Failed | - | Hartmann | No |
| 11 (M/45) | Primary Ca descending | Descending | Temporizing | Successful | 1 | Left colectomy | Migration |
| 12 (F/70) | Primary Ca rectum | Upper rectum | Temporizing | Successful | 1 | AR | No |
| 13 (M/48) | Recurrent Ca rectum | Upper rectum | Palliative | Successful | 1 | Chemo-radio therapy | Anal pain |
| 14 (M/72) | Primary Ca descending | Splenic flexure | Temporizing | Successful | 1 | Left colectomy | No |
| 15 (F/63) | Primary Ca sigmoid | Sigmoid | Temporizing | Successful | 1 | Sigmoidectomy | Migration |
| 16 (M/40) | Primary Ca rectum | Upper rectum | Temporizing | Successful | 1 | LAR | No |
| 17 (M/72) | Primary Ca descending | Descending | Temporizing | Successful | 1 | Hartmann | Food residue obstruction |
| 18 (F/79) | Primary Ca rectum Liver metastasis | Middle rectum | Palliative | Successful | 1 | Chemotherapy | No |
| 19 (M/45) | Primary Ca descending | Descending | Temporizing | Successful | 1 | Left colectomy | No |
| 20 (M/85) | Primary Ca descending | Descending | Temporizing | Successful | 1 | Left colectomy | No |
| 21 (M/41) | Gastric Ca rectum seeding | Rectum | Palliative | Successful | 1 | Chemo-therapy | No |
| 22 (M/69) | Primary Ca descending | Descending | Temporizing | Failed | - | Hartmann | No |
| 23 (M/74) | Primary Ca sigmoid | Sigmoid | Temporizing | Successful | 1 | Sigmoidectomy | No |
| 24 (M/85) | Primary Ca sigmoid | Sigmoid | Temporizing | Successful | 1 | Sigmoidectomy | No |
| 25 (M/75) | Primary Ca sigmoid | Sigmoid | Temporizing | Successful | 1 | Sigmoidectomy | No |
| 26 (F/67) | Primary Ca sigmoid | Sigmoid | Temporizing | Successful | 1 | Sigmoidectomy | No |

the stent was considered as a definitive palliative treatment. In only one case the abdominal computed tomography scan failed to detect peritoneal implants of tumor, and the metastatic disease was identified at the time of laparotomy. As a result, 17 (94%) out of 18 unnecessary operations were avoided^[24].

It was also reported that patients who were not operated have a shorter hospital stay and lower incidence of stoma after SEMS placement^[25].

Binkert *et al.*^[26] showed that SEMS placement can

reduce the cost due to a shorter hospital stay and a lower complication rate. Morino *et al.*^[27] have proposed a new minimally invasive therapeutic strategy for the management of acute malignant colonic obstructions: emergency endoscopic stenting followed by elective laparoscopic one-stage resection. They reported that four patients with malignant colonic obstruction had an immediate restored bowel functions after SEMS deployment within a period that varied from 4 to 5 days, then underwent a one-stage laparoscopic resection and were discharged 5-7 days later

without any complications.

Preoperative stent insertion for obstructive colorectal cancer can result in satisfactory postoperative outcome with good prognoses^[28]. Perforation is the most severe complication of SEMS placement. Neoplastic strictures of the colon are generally soft. Acute neoplastic occlusion may be caused by neoplastic edema or stool residue or both. If the guide wire can be passed through neoplastic strictures, stent placement without balloon dilation should be easy for experienced physicians. The incidence of perforation was 2% (12/493) in the non-balloon dilatation group and 10% (10/105) in the dilatation group, suggesting that balloon dilation should not be recommended^[9].

Some minor SEMS-placement-related complications including stent migration, mild bleeding, pain, and reobstruction may occur. Stent migration occurred in two patients of our group possibly due to abated edema and/or inadequate stent. Because of the limited cases and short survival time, we did not find reobstruction.

In conclusion, colorectal stenting procedure is effective and safe and can be used for acute left-sided colorectal malignant obstruction both as a temporary relief before selective resection and as a definitive treatment in palliative cases.

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CLINICAL RESEARCH

Objective assessment of the antispasmodic effect of Shakuyaku-kanzo-to (TJ-68), a Chinese herbal medicine, on the colonic wall by direct spraying during colonoscopy

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assessment

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Abstract

AIM: To objectively evaluate the effect of TJ-68 on colonic spasms during colonoscopy.

METHODS: One hundred and one patients subjected to screening colonoscopy were randomly assigned to two groups: TJ-68 in 51 subjects and saline as the control in 50. The endoscope was inserted into the sigmoid colon, then a spastic region was identified and the tip of the colonoscope was positioned at a distance of about 10 mm from the spastic region. The endoscopic view was recorded before and after direct spraying of the TJ-68 solution or warm saline. The intraluminal area of the spastic region was serially measured using a computer image analyzer and expressed as pixel counts. The area under the curve (AUC) was calculated from the pixel curve. Statistical significance was assessed by Wilcoxon's test and Mann-Whitney *U* test.

RESULTS: The mean AUC of the spastic region before and after TJ-68 spraying was 29 128 and 121 943 pixels, respectively, while with saline, it was 31 635 pixels and 48 617 pixels, respectively. Thus, the AUC significantly increased after TJ-68 spraying compared with the spraying of saline ($P < 0.001$).

CONCLUSION: Direct spraying of TJ-68 on the colonic mucosa suppressed colonic spasm and it may be useful during colonoscopy when anticholinergic agents are contraindicated.

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Key words: Colonoscopy; Antispasmodic effect; Objective

INTRODUCTION

In colonoscopy, it is often difficult to insert the endoscope and observation tends to be hampered because of colonic spasms. In patients with colonic spasm sometimes excess air is used to get a good view of the colonic mucosa causing discomfort and pain in the patient, and resulting in an unsuccessful colonoscopy.

Hyoscine *n*-butyl bromide (Hyoscine) is usually and widely used as an antispasmodic agent in colonoscopy and it is administered by intramuscular or intravenous injection. However, systemic use of Hyoscine sometimes causes many adverse reactions; namely palpitation, dry mouth, urinary retention, orthostatic hypotension, anaphylactic reactions, and temporary visual impairment. Hyoscine, in addition, often causes serious complications in patients with heart disease and it is contraindicated in such cases. For this reason, a safer means to obtain the necessary antispasmodic effect is required for colonoscopy. In this regard, peppermint oil and warm water have been investigated by directly spraying them onto the colonic wall, however, there are some limitations for their clinical use^[1,2].

Shakuyaku-kanzo-to (TJ-68), a Chinese herbal medicine, is traditionally used in China and Japan, to treat patients with convulsions, spasms, a pain in the limb, etc. Recently, the analgesic and sedative effects of TJ-68 as a premedication have been reported in endoscopy^[3]. Although the antispasmodic effects of TJ-68 during barium enema examination was studied, the usefulness of TJ-68 as an antispasmodic agent in colonoscopy has not been examined^[4].

The aim of this study was to objectively evaluate

Table 1 Background characteristics of the patients

| | TJ-68 | Control |
|-----------------------------|-----------|-----------|
| No. of cases | 51 | 50 |
| Male/female | 34/17 | 35/15 |
| Age (yr) (mean±SD) | 60.0±14.8 | 61.0±11.7 |
| Indication of colonoscopy | | |
| Polyp surveillance | 15 | 13 |
| Post EMR surveillance | 0 | 3 |
| Fecal occult blood positive | 13 | 6 |
| Abdominal pain | 3 | 6 |
| Constipation | 4 | 3 |
| Diarrhea | 2 | 5 |
| Anemia | 1 | 0 |
| Screening | 5 | 5 |
| Hematochezia | 4 | 6 |
| Bloody stool | 4 | 2 |
| Soft stool | 0 | 1 |

¹NS *vs* control (Mann-Whitney *U* test), ²*P*<0.001 *vs* before (Wilcoxon's test), ³*P*<0.001 *vs* control after (Mann-Whitney *U* test), ⁴NS *vs* before (Wilcoxon's test).

the antispasmodic effect of TJ-68 by direct intraluminal spraying of TJ-68 solution during colonoscopy.

MATERIALS AND METHODS

Patients

A total of 531 patients were registered for endoscopy between July 2002 and March 2004. Exclusion criteria were as follows: suspected intestinal bleeding, severe abdominal pain accompanied by acute colitis and inflammatory bowel disease, history of abdominal surgery, and habitual user of drugs which may affect bowel motility. Of these, 110 patients who fulfilled the eligibility criteria were allocated at random to either the TJ-68 group or the control (saline) group. Of them, 9 patients - 5 in the TJ-68 group and 4 in the control group - were excluded from this study: due to loss of sight of the endoscopic view because of influx of residual irrigation solution from the oral side. The background characteristics of all the subjects are shown in Table 1. There was no significant difference in gender, mean age, or the reason for colonoscopy between the two groups.

Methods

This study was a prospective, randomized trial performed from July 2002 to March 2004 to compare the effects of TJ-68 solution (Tsumura Co., Tokyo, Japan) with those of saline, administered by intraluminal spraying on a colonic spastic area during colonoscopy. Colonoscopy was performed by one endoscopist (MA) using a standard colonoscope (CF-Q240I; Olympus Optical Co., Ltd, Tokyo). All patients were given a routine bowel preparation including 59 g of magnesium citrate in 250 mL (Magcorol P, Horii Pharmaceutical Ind., Ltd, Osaka, Japan) on the day before the procedure and ingestion of 2 L of a balanced solution of electrolytes with 137.155 g of polyethyleneglycol (Niflec, Ajinomoto Pharma Co., Ltd, Tokyo) before the procedure. The TJ-68 solution contained; 0.5 g of TSUMURA Shakuyaku-kanzo-to

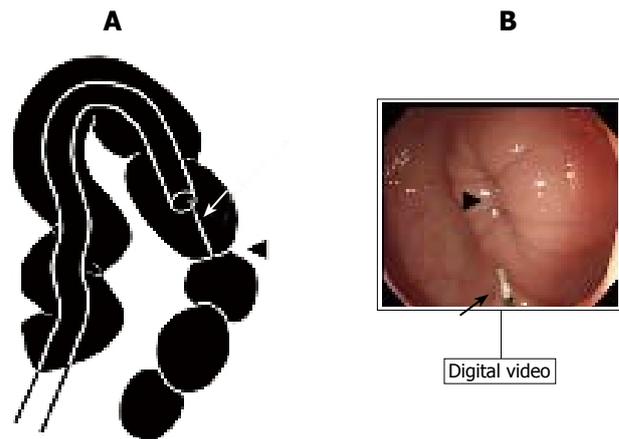


Figure 1 Illustration of the procedure (A) and actual endoscopic image (B). A distance of about 10 mm was kept between the spastic region (arrow head) and the tip of the colonoscope. The distance was objectively measured using a wire (arrow) which was inserted through the working channel.

Extract Granules for Ethical Use dissolved in 50 mL saline (10^{-2} g/mL) at 36 °C. Normal saline was also prepared at 36 °C. In our experimental observations, we found that this concentration of TJ-68 solution significantly relaxed guinea pig colonic smooth muscle (unpublished), therefore we used TJ-68 at this concentration in the present study. During the procedure, we did not use any sedative agents.

With the patient in the left decubitus position, the endoscope was advanced to about 20-25 cm from the anal canal into the sigmoid colon and then, a spastic region was identified. With the tip of the colonoscope positioned at about 10 mm from the surface of the spastic area, endoscopic views were recorded for 6 min on digital videotape before and after spraying TJ-68 solution or saline through a working channel. During the examination, no air was used. Distance between the region and the tip of the endoscope was confirmed regularly using a measured guide-wire (Figure 1).

Informed consent was obtained from all the patients and the protocol was reviewed and approved by the Institutional Review Board of our hospital.

Measurement of the intraluminal spastic area

On the personal computer (VAIO PCG-GR5F, Sony), 12 spot images (3.0, 2.5, 2.0, 1.5, 1.0, and 0.5 min before and after spraying the solution) were captured every 30 s to assess luminal changes using a computer software (DVgate Still, Sony) from the recorded digital videotapes. The intraluminal spastic area was measured using a computer image analyzer (Adobe Photoshop 7.0, Adobe Systems Co., San Jose, CA, USA) and expressed as pixel counts (Figure 2). Then the mean pixel counts were used to construct a pixel curve in each group. The mean area under the curve (AUC) was calculated from the pixel curve obtained before and after spraying the TJ-68 solution or saline in each group.

Statistical analysis

Normally distributed continuous data are presented as the mean plus standard deviation and differences between the



Figure 2 The white broken line demarcates the intraluminal spastic region and the area of the region was measured using a computer image analyzer (*).

Table 2 Comparison of the mean AUC between the TJ-68 and control groups before and after spraying of TJ-68 or saline

| | Mean AUC (pixels × min) | |
|---------|-------------------------|------------------------|
| | Before | After |
| TJ-68 | 29 127 ¹ | 121 942 ^{b,d} |
| Control | 31 635 | 4 8617 ² |

¹NS vs control (Mann-Whitney U test), ^b $P < 0.001$ vs before (Wilcoxon's test), ^d $P < 0.001$ vs control after (Mann-Whitney U test), ²NS vs before (Wilcoxon's test).

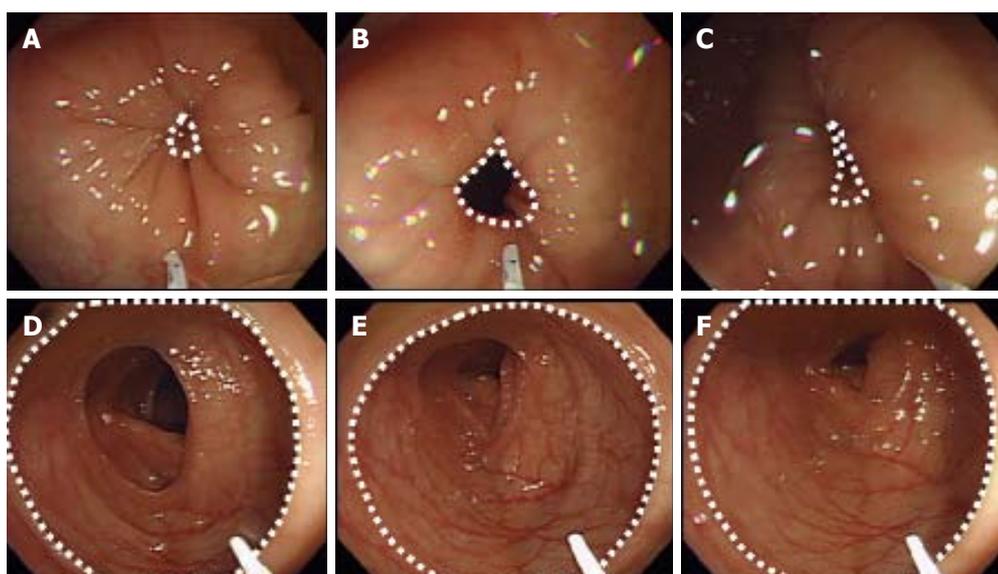


Figure 3 Endoscopic view in the TJ-68 group. Serial endoscopic views of the spastic region at the sigmoid colon before and after spraying of the TJ-68 solution are shown (A-F). The white broken line demarcates the area of the spastic region. The spastic region started to relax 0.5 min after spraying of the TJ-68 solution and remained open for 3 min.

groups were evaluated by Student's *t* test. Categorical data were compared with the χ^2 test and Fisher's exact test.

Wilcoxon's test was used to compare the AUCs before and after spraying the TJ-68 solution or saline in each group. Mann-Whitney *U* test was used to compare the AUCs before or after spraying. A *P* value of < 0.01 was considered statistically significant. Data were analyzed using a statistical software package (Stat Mate III, ATMS Co., Ltd, Tokyo).

RESULTS

Representative endoscopic photographs of the spastic area in the sigmoid colon taken from one patient in each group before and after spraying of TJ-68 solution and normal saline are shown in Figures 3 and 4.

Antispasmodic effects of the TJ-68 solution

The mean changes of intraluminal spastic area before and after spraying of the agents are shown in Figure 5. In the TJ-68 group, the intraluminal area varied from 8 954 to 13 181 pixels at each point during 3 min before spraying, showing no difference, but after spraying, an obvious increase was observed. The intraluminal area rapidly increased 1 min after spraying and the mean area 3 min after it was 56 063 pixels. The mean AUC before and after TJ-68 spraying was 29 127 and 121 942 pixels/min,

respectively, showing significant difference ($P < 0.001$).

The mean intraluminal spastic area in the control group varied from 10 696 to 15 246 pixels at each point during 3 min before spraying, showing no difference. Spraying of saline did not significantly affect the mean intraluminal spastic area, and there was no significant increase of the mean AUC after spraying: 31 635 and 48 617 pixels/min, respectively. Thus, the AUC significantly increased after TJ-68 spraying compared with the spraying of saline ($P < 0.001$, Table 2).

No complication was encountered in this study.

DISCUSSION

The TJ-68 solution caused the relaxation of the spastic colonic region as early as 0.5 min after spraying, and the relaxation was maintained for 3 min, or more. This phenomenon was sought to be as the local effects of the TJ-68 on the colonic wall. While in the control, the spastic area did not increase significantly after spraying of saline. Judging from the increase in the number of pixels in the control, this increase seemed to be simply attributable to a volume load. While the increase in the TJ-68 group was attributable to the active relaxing effect of TJ-68, and relaxation of the spastic area was observed within 0.5 min after spraying. We think there is a close correlation between the intraluminal movement of a spastic region

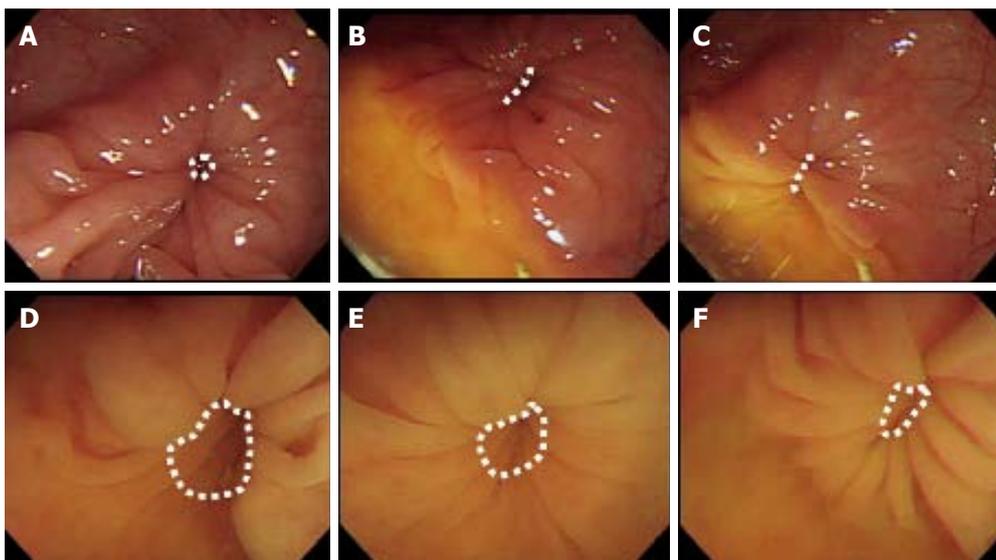


Figure 4 Endoscopic view in the normal saline group. Serial endoscopic views of the spastic region at the sigmoid colon before and after spraying of normal saline are shown (A-F). The white broken line demarcates the area of the spastic region. The region showed only little relaxation and did not open significantly during the study.

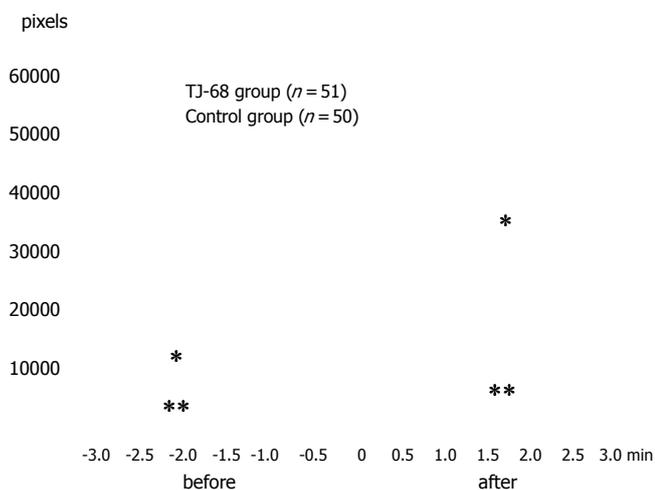


Figure 5 Pixel curve and area under the curve (AUC) before and after the administration of the TJ-68 solution and normal saline. The graph shows the mean AUC of the two groups before and after spraying of TJ-68 (*) and normal saline (**): the mean AUC in the TJ-68 group increased significantly from 29 127 to 121 942 pixels, but the control group did not show a significant increase.

and the intraluminal pressure. Actually, Asao *et al.* reported that the changes of the endoscopic findings by the intraluminal administration of the peppermint oil solution is correlated well with the intraluminal pressure measured by the Barostat^[1]. Based on this phenomenon, we assume that TJ-68 will permeate and be absorbed through the colonic wall, and directly act on the myenteric plexus.

Since TJ-68 solution has a light brown color, blinding of the study is thought to be difficult in the strict sense. But selection of the patients or assignment of the agents to each group was performed in a strictly blind fashion. Furthermore, as relaxation of spasms was evaluated objectively, we believe that discernment of the agent after spraying had only a little influence on the outcome.

TJ-68 is an extract powder composed of Shakuyaku (*Paeoniae radix*) and Kanzo (*Glycyrrhizae radix*) combined at a ratio (g) of 1:1. TJ-68 inhibits acetylcholine-induced contraction and the contractile machinery of the smooth

muscle by Kanzo and that of neurogenic contraction by Shakuyaku in guinea pig ileum^[5,6]. Furthermore, the two components of TJ-68 exert synergistic effects^[5].

Peppermint oil and warm water were examined in a way, analogous to our study, for their relaxing effect on the colonic wall after direct spraying^[1,2]. Peppermint oil was reported to have a direct relaxing effect on the colonic smooth muscle when used in endoscopy or barium enema examination^[1,7,8]. We also studied the antispasmodic effect of peppermint oil on the colonic wall using our method of evaluation and compared it to that of TJ-68. As a result, TJ-68 was found to have an antispasmodic effect similar or stronger than that of peppermint oil (data not shown). Peppermint oil is not sold via a regular medical distribution system. Furthermore, it is sometimes difficult to get and prepare the peppermint oil solution^[1]. On the other hand, TJ-68 is available in any medical facility in Japan, because it is a regular medical prescription sold via a regular medical distribution system. In addition, the preparation of TJ-68 solution is easy and simple: only mixing TJ-68 with saline.

Church reported that warm water irrigation significantly reduced discomfort related to colonoscopy, but there was no difference regarding insertion time, total duration of colonoscopy, dose of midazolam administered, or frequency of severe spasm. Decrease of the patient's discomfort was the parameter of effectiveness of warm water in their study. But their study had some limitations because they used a sedation drug (midazolam) as premedication^[2]. Moreover, warm water has basically no direct antispasmodic effect on the colonic wall.

Colonic relaxation by antispasmodic drugs has been evaluated using various parameters: pain score, colonic spasm score, difficulty of the procedure scored by an endoscopist, and dose of drugs used for sedation^[1,2,9-14]. However, these parameters were considered to be subjective, moreover, most studies were performed under sedation^[2,9-14]. In the present study, we evaluated colonic relaxation quantitatively. In addition, we did not use any drugs to induce sedation. But the clinical relevance of TJ-68 in colonoscopy should be investigated in subsequent studies, although our preliminary study showed significant

differences between the TJ-68 group and the control group in total colonoscopy time and patient's pain score (data not shown).

Contrary to the ordinary use of antispasmodic agents, we sprayed TJ-68 directly on the colonic wall. Antispasmodic agents are generally administered intramuscularly or intravenously, but systemic administration sometimes prevents the insertion of the endoscope because the muscle tone of the colon is excessively reduced^[13]. Colonoscopy is difficult to carry out at the spastic region but not every spasm affects the examination. Ideally, the examiner expects to obtain effective relaxation in only some of them. In this respect, direct spraying of the antispasmodic agent TJ-68 is one of the meaningful ways of administration. But administration of this agent by spraying differed from the common oral administration. Since this was the first attempt to spray this agent directly onto the colonic wall, we had essentially no idea about the adverse effects. But the total amount of TJ-68 used in this study was no more than 2.5 g, that is to say one third of the oral dose: 7.5 g. In fact, no complication was experienced in this study.

In this study, we set the concentration of the TJ-68 solution at 10^{-2} mg/dL. Significant relaxation of the guinea pig distal colon was obtained with this concentration of TJ-68 in our preliminary experimental study; thus this concentration was thought to be effective enough to depress colonic spasm during colonoscopy.

In conclusion, we objectively demonstrated that intraluminal direct spraying of TJ-68 solution on the colonic mucosa could lower the colonic spasm. Additional studies are needed to determine the time required for colonoscopy or to assess patient's compliance before TJ-68 can be definitively recommended as a useful antispasmodic agent for use in colonoscopy.

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Surgical treatment of patients with intermediate-terminal pancreatic cancer

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Abstract

AIM: To investigate the surgical treatment of patients with intermediate-terminal pancreatic cancer.

METHODS: A retrospective analysis was made of the clinical data of 163 patients with intermediate-terminal pancreatic cancer who were surgically treated between August 1994 and August 2003.

RESULTS: A total of 149 patients underwent palliative surgery. The mortality rate of those who underwent cholecystojejunostomy alone was 14.2%, the icterus or cholangitis recurrence rate was 61.9% with an average survival period of 7.1 mo. The mortality rate for those who received hepatic duct-jejunostomy (HDJS) was 5.7%, the icterus or cholangitis recurrence rate was 6.8% with an average survival period of 7.1 mo. But 31.8% of the patients developed duodenum obstruction within 6 mo after the surgery, six of seven patients with severe pain were given peri-abdominal aorta injection with absolute alcohol and their pain was alleviated. The other patients underwent percutaneous transhepatic cholangial drainage (PTCD) and their icterus index returned to normal level within 40 d with an average survival period of 7.5 mo.

CONCLUSION: Roux-en-y HDJS combined with prophylactic gastrojejunostomy is recommended for patients with intermediate-terminal pancreatic cancer, and biliary prosthesis can partly relieve biliary obstruction in a short term.

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Key words: Pancreatic cancer; Bile duct conduction; Gastrointestinal conduction; Biliary prosthesis

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INTRODUCTION

Generally speaking, not until the patient has developed terminal pancreatic cancer can a diagnosis be made. The non-clinical survival time is not more than half a year and the post-operative survival rate is still lower than 20%^[1]. At present, surgical treatment is the most common method for patients with intermediate-terminal pancreatic cancer. The hospital received 163 patients with intermediate-terminal pancreatic cancer between August 1994 and August 2003.

MATERIALS AND METHODS

Basic data

A total of 107 male and 56 female patients aged 28-79 years with an average age of 43.2 years were included in the study.

Clinical symptoms

The most common symptoms were upper-abdominal spasm and gastric bulge without any motivation. One hundred and twelve patients (68.7%) suffered from anorexia and only 51 patients (31.2%) developed icterus. Among these 163 patients, 124 were not in the hospital until they had icterus. All the 163 patients were diagnosed as intermediate-terminal pancreatic cancer by B-ultrasound, CT, MR and explorative operation.

Operation procedures

One hundred and forty-nine patients underwent palliative surgery. Among them, 28 patients received cholecystojejunostomy (CJS), 53 hepatic duct-jejunostomy (HDJS) and 61 biliary enteric anastomosis plus gastrojejunostomy, 7 terminal peri-abdominal aorta injection with absolute alcohol. The other 14 patients did not undergo surgery but biliary prosthesis placement for PTCD.

RESULTS

Among the 149 patients who received palliative surgical treatment, 10 died 30 days after the surgery. The estimated

mortality rate of those who underwent CJS, HDJS, and biliary enteric anastomosis plus gastrojejunostomy was 14.2% (4/28), 5.7% (3/53), and 4.9% (3/61), respectively. Among the 21 patients who underwent CJS, 13 developed icterus or cholangitis, the recurrence rate was 61.9% with an average survival period of 7.1 mo. Among the 44 patients who underwent hepaticocholechojejunostomy, only 3 developed icterus or cholangitis, the recurrence rate was 6.8% with an average survival period of 10.3 mo. Likewise, the recurrence rate of icterus or cholangitis among the 51 patients who underwent biliary enteric anastomosis plus gastrojejunostomy was 5.9% (3/51), but the average survival period was 13.9 mo.

Fourteen out of the forty-four patients who were followed up after HDJS developed duodenal obstruction, the rate was 31.8%. Among them, 11 patients who received an additional gastrojejunostomy did not survive for more than 3 mo. Fifty-one patients who received biliary enteric anastomosis in combination with gastrojejunostomy needed no additional surgical treatment for duodenal obstruction.

The icterus index of 14 patients became normal within 40 days after biliary prosthesis placement, 11 followed-up cases had an average survival period of 7.5 mo. Seven patients were given absolute alcohol injection and their pain was relieved with an average survival period of 3.4 mo.

DISCUSSION

Surgical treatment of patients with intermediate-terminal pancreatic cancer

Most patients have already developed terminal pancreatic cancer when a confirmed diagnosis was made. Palliative surgical treatment is the first choice of treatment for these patients. The purposes of the treatment are to relieve biliary tract and duodenal obstruction, to control and alleviate the symptoms in order to prolong the post-operative survival time^[2].

Common treatment approaches for intermediate-terminal pancreatic cancer include biliary enteric anastomosis, biliary prosthesis, and Roux-Y loop, etc. Biliary enteric anastomosis including cholecystojejunostomy (CJS) and HDJS is the most commonly used approach today.

CJS is easy to perform and does not incur severe trauma, but has certain disadvantages. For example, 10% of the patients have the opening of the cystic duct on the lower part of the common bile duct, thus the patients are liable to develop duodenal obstruction due to tumors and metastatic lymph nodes. Since it is difficult to make sure whether the bile duct is blocked during surgery, cholangiography is needed, thus the surgery is time-consuming. Such a surgery is inappropriate in the presence of cholecystitis, gallstones or ill-function. Post-operative icterus and cholangitis are more likely to occur. Rosemurgy *et al*^[4] demonstrated that the success rate of CJS and HDJS to relieve icterus is 89% and 97%, respectively, and the incidence rate of icterus and cholangitis is 20% and 8%, respectively. In our patients, the recurrence rate of icterus or cholangitis was 61.9%, much higher than that after HDJS (6.8%). The average survival period (7.1 mo) was far lower than that after HDJS (10.3 mo), suggesting that HDJS is a

better therapeutic approach than CJS.

It was reported that Roux-en-Capital Y anastomosis can be performed for patients with pancreatic cancer, because the suturing position of HDJS is relatively higher and less liable to be assaulted by tumors and suturing point is larger^[4]. On the other hand, Roux-en-y anastomosis can significantly reduce the incidence of reflux cholangitis and hepatitis^[6]. In addition, HDJS incurs direct hepatobiliary drainage, thus effectively reducing the incidence of icterus as well as the recurrence rate of icterus or cholangitis^[6]. We believe that it is suitable for patients whose potential survival period is relatively longer. However, the common bile duct in the superior border of the duodenum should be cut off leaving the distal end blocked, and the proximal end should be anastomosed to the duodenum to avoid tumor-incurred icterus. The common bile duct should be sutured with only one layer of stitches and the passage above the stitches should be unobstructed.

Biliary tract drainage or percutaneous transhepatic biliary drainage (PTBD) can effectively reduce icterus. But loss of bile seriously unbalances the electrolytes.

Pre-emptive application of gastrojejunostomy and pancreaticojejunostomy

One of the most important problems in the treatment of pancreatic cancer is whether the surgeon can resolve the duodenal obstruction during the biliary enteric looping process. It was reported that apart from direct tumor assaults, gastrointestinal dysfunction caused by visceral nerves is also a reason for duodenal obstruction^[7]. When duodenal obstruction occurs, GJS cannot improve the gastric drainage^[8]. Therefore pre-emptive GJS is not recommended. But 13-34% of the patients undergoing biliary enteric anastomosis suffer from duodenal obstruction^[6]. An additional GJS can prolong the survival period significantly but does not increase the mortality rate. Gastrointestinal conduction can assist the treatment by providing nutrition. The data in this series also showed that compared to biliary enteric anastomosis only, biliary enteric anastomosis combined with GJS resulted in a sharp increase of mortality rate but prolonged the post-operative survival time. Besides, biliary enteric anastomosis alone tended to cause duodenal obstruction, while the combination reduced its occurrence, suggesting that GJS should be normally accepted as an approach for unresectable patients. This is because terminal pancreatic cancer patients are more liable to develop duodenal obstruction, the mortality rate is high and the survival time is short when GJS is performed after the diagnosis of duodenal obstruction. Biliary enteric anastomosis in combination with GJS does not increase the risk of operative deaths, while prolongs the post-operative survival time.

It was reported that biliary enteric anastomosis combined with pancreatoenterostomy can prevent duodenal or pancreatic obstruction, relieve the accompanying pain caused by dilation and indigestion, and reduce the mortality rate^[10]. We believe that patients with apparent indigestive symptoms and pancreatic dilation confirmed by ultrasonic and CT examinations can undergo pancreatoenterostomy, since three anastomoses can be made in one surgery and there is no need for subsequent surgeries.

Application of biliary prosthesis

Biliary prosthesis is an advanced technique and the trauma caused by it is small, thus, patients can recover quickly. In our study, 14 patients with terminal pancreatic cancer underwent PTCD and their icterus index returned to normal level within 40 d, suggesting that this operation is applicable to terminal pancreatic cancer patients or very old patients in bad physical conditions. But this new technique leads to some complications, such as a relatively high failure rate and no long-term effect on reducing icterus. It is suggested that surgical treatment can reduce icterus in patients and prolong post-surgery survival^[2].

Treatment of persistent pain

Persistent pain is one of the major symptoms of intermediate-terminal pancreatic cancer patients, 30-40% of the patients suffer from abdominal and back pain due to cancer-afflicting post-peritoneum nerve plexus or chronic pancreatitis. In addition, high pressure of biliary tract, cholangitis or incomplete duodenal obstruction can also be a factor. Pain can be relieved or partly relieved by biliary or gastrointestinal by-pass surgery for some patients. But they have no effect on pain caused by post-peritoneum nerve plexus affliction. Some scholars have proposed that using a dose of 20 mL alcohol (50%) or lidocaine (1%) as a blockage injection to post-peritoneum nerve plexus near the tumor can alleviate or prevent late pancreatic cancer-related pain.

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RAPID COMMUNICATION

Portal vein pulsatility index is a more important indicator than congestion index in the clinical evaluation of right heart function

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CONCLUSION: Our data show that RI is a more significant indicator than CI in the clinical evaluation of high RA ≥ 10 mmHg, whereas CI is better than PI in the assessment of left heart function.

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Key words: Portal blood flow; Heart failure; Ultrasonic Doppler; Congestion index; Portal vein pulsatility index

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Abstract

AIM: To study the changes of portal blood flow in congestive heart failure.

METHODS: We studied the congestion index (CI) and portal vein pulsatility index (PI) in patients with varied degrees of congestive heart failure using ultrasonic Doppler. Ten patients with mean right atrial pressure (RA) < 10 mmHg were classified as group 1 and the remaining 10 patients with RA ≥ 10 mmHg as group 2.

RESULTS: There were no difference on cardiac index (HI, $P=0.28$), aortic pressure (AO, $P=0.78$), left ventricular end-diastolic pressure (LVED, $P=0.06$), maximum portal blood velocity (V_{max} , $P=0.17$), mean portal blood velocity (V_{mean} , $P=0.15$) and portal blood flow volume (PBF, $P=0.95$) between the two groups. Group 2 patients had higher pulmonary wedge pressure (PW, 29.9 ± 9.3 mmHg vs 14.6 ± 7.3 mmHg, $P=0.002$), pulmonary arterial pressure (PA, 46.3 ± 13.2 mmHg vs 25.0 ± 8.2 mmHg, $P=0.004$), RA (17.5 ± 5.7 mmHg vs 4.7 ± 2.4 mmHg, $P < 0.001$), right ventricular end-diastolic pressure (RVED, 18.3 ± 5.6 mmHg vs 6.4 ± 2.7 mmHg, $P < 0.001$), CI (8.7 ± 2.4 vs 5.8 ± 1.2 , $P=0.03$), and PI ($87.8 \pm 32.3\%$ vs $27.0 \pm 7.4\%$, $P < 0.001$) than Group 1. CI was correlated with PI ($P < 0.001$), PW ($P < 0.001$), PA ($P < 0.001$), RA ($P=0.043$), RVED ($P=0.005$), HI ($P < 0.001$), AO ($P < 0.001$), CO ($P < 0.001$), LVED ($P < 0.001$), V_{max} ($P < 0.001$), V_{mean} ($P < 0.001$), cross-sectional area of the main portal vein ($P < 0.001$) and PBF ($P < 0.001$). CI could be as high as 8.3 in patients with RA < 10 mmHg and as low as 5.9 in those with RA ≥ 10 mmHg.

INTRODUCTION

Congestive heart failure increases the pressure in the inferior vena cava and hepatic veins^[1-3]. Ultrasonic Doppler is a safe and non-invasive method in the clinical evaluation of portal blood flow and portal hypertension^[4-9]. Portal vein pulsatility index (PI) is calculated by the percentage of peak-to-peak maximum portal vein velocities^[10,11]. In our earlier study^[10], patients with right heart failure developed transient reduced, stagnant, or hepatofugal portal blood flow with increased PI. However, the change of portal flow pattern and PI did not correlate with left heart function.

The congestion index (CI) has been used to assess the pathophysiological hemodynamics of portal venous system in different forms of liver diseases^[12-14]. The correlation between CI and PI and the role of CI on right heart function remain uncertain. Therefore, we have studied the changes of portal blood flow in patients with different degrees of heart failure using non-invasive ultrasonic Doppler^[15,16].

MATERIALS AND METHODS

We studied the portal hemodynamic profiles in 20 patients (9 males, 11 females, mean age: 49 ± 13 years) who received cardiac and Swan-Ganz catheterizations for cardiovascular disorders (16 rheumatic heart disease, 4 atherosclerotic heart disease) to compare with 20 healthy volunteers. All

Table 1 Clinical and biochemical data in patients with heart failure (mean \pm SD)

| | Controls | RA < 10 mmHg | RA \geq 10 mmHg |
|--------------------------------|---------------|---------------|---------------------------|
| Gender (M/F) | 10/10 | 4/6 | 5/5 |
| Age (yr) | 46 \pm 12 | 50 \pm 13 | 47 \pm 19 |
| Total protein (g/dL) | 7.5 \pm 0.6 | 7.1 \pm 0.8 | 6.9 \pm 1.1 |
| Albumin (g/dL) | 4.3 \pm 0.2 | 3.9 \pm 0.7 | 3.7 \pm 0.5 |
| Total serum bilirubin (mg/dL) | 0.9 \pm 0.4 | 1.3 \pm 0.8 | 1.4 \pm 0.8 |
| AST (U/L) | 21 \pm 6 | 31 \pm 11 | 59 \pm 29 ^{1b} |
| ALT (U/L) | 24 \pm 6 | 23 \pm 8 | 35 \pm 20 |
| Prolonged prothrombin time (s) | - | 1.1 \pm 0.9 | 1.4 \pm 0.8 |

RA, right atrial pressure; ¹ $P=0.009$ vs patients with RA < 10 mmHg and ² $P<0.001$ vs controls.

Table 2 Cardiac profiles in patients with congestive heart failure (mean \pm SD)

| | RA < 10 mmHg | RA \geq 10 mmHg | <i>P</i> |
|----------------------------|----------------|-------------------|----------|
| HI (L/min/m ²) | 3.0 \pm 0.9 | 2.4 \pm 0.4 | 0.28 |
| AO (mmHg) | 89.0 \pm 9.6 | 87.3 \pm 12.8 | 0.78 |
| LVED (mmHg) | 12.2 \pm 6.7 | 22.1 \pm 10.9 | 0.06 |
| PW (mmHg) | 14.6 \pm 5.6 | 29.1 \pm 7.7 | 0.002 |
| PA (mmHg) | 25.0 \pm 6.8 | 42.4 \pm 12.0 | 0.004 |
| RA (mmHg) | 4.7 \pm 2.1 | 16.8 \pm 4.9 | <0.001 |
| RVED (mmHg) | 6.4 \pm 2.1 | 17.8 \pm 4.4 | <0.001 |

RA, right atrial pressure; HI, cardiac index; LVED, left ventricular end-diastolic pressure; AO, mean aortic pressure; PW, pulmonary wedge pressure; PA, mean pulmonary arterial pressure; RA, mean right atrial pressure; RVED, right ventricular end-diastolic pressure.

patients had medications affecting the hemodynamics such as isosorbide dinitrate and furosemide, and their systemic blood pressure and body weight were measured to be constant for more than 48 h prior to the study. Patients with fever, infection, and shock were excluded. All patients had no history of liver disease, alcoholism or other metabolic disorders. None of the patients received transfusion, inotropic agents or dopamine. All patients had an abdominal sonography to exclude chronic liver disease or splenomegaly. Patients with severe orthopnea were excluded if they were not able to remain in the supine position for the study of ultrasonic Doppler.

Cardiac profiles including cardiac index (HI), left ventricular end-diastolic pressure (LVED), mean aortic pressure (AO), pulmonary wedge pressure (PW), mean pulmonary arterial pressure (PA), mean right atrial pressure (RA), right ventricular end-diastolic pressure (RVED) were recorded during the cardiac and Swan-Ganz catheterizations. Ten patients with RA < 10 mmHg (range: 1-7 mmHg) and without right heart failure were classified as Group 1. The remaining 10 patients with right heart failure and RA \geq 10 mmHg (range: 10-28 mmHg) were classified as Group 2.

The portal profiles were assessed using an ultrasonic Doppler composed of a real-time mechanical sector scanner and a 3.5 MHz pulsed Doppler flowmetry (Aloka Echo Camera, Model SSD-1700, Tokyo) within 12 h of cardiac catheterization. After more than 8 h of fasting, portal pro-

files were measured in the supine position for more than 30 min. Portal blood flow was measured from the main portal vein at a site just entering or immediately after entering the liver with the patient in expiratory apnea. The flow angle formed by the directions of ultrasonic beam and the portal blood flow below 55 degree was corrected to minimize the variation caused by the angle of insonation. The Doppler signal could be viewed on the screen and heard through a build-in speaker. Portal blood flow was measured by the same physician (SY) to avoid interobserver variation^[17].

For each measurement, at least three reproducible spectral patterns were recorded for calculating the mean maximum portal blood velocity (V_{max}) over a period of 3-4 s to ensure accuracy. Mean portal blood velocity (V_{mean}) was calculated by the equation " $V_{mean}=0.57 \times V_{max}$ " as described by Moriyasu *et al.*^[18]. Cross-sectional area (area, cm²) was also recorded at the site of main portal vein where portal blood velocity was measured. The direction of portal blood velocity, antegrade or retrograde, was also measured. Positive velocity indicates the blood flow towards the transducer and vice versa. Portal blood flow volume (PBF, mL/min) was obtained by the equation " $PBF=area \times V_{mean} \times 60$ "^[17,18]. PI was calculated by the equation " $PI=(\text{maximum}-\text{minimum})/\text{maximum frequency shift}$ "^[6,15,17]. The waveforms were classified as continuous (PI \leq 40%), decreased (PI 41-99%), stagnant (PI = 100%), or retrograde (PI > 100%)^[10,19]. CI was calculated by the equation " $CI=(area/V_{mean}) \times 100$ "^[12].

The study protocol was reviewed and approved by the Institutional Review Committee under the guidelines of the 1975 Declaration of Helsinki. Statistical analysis was performed using Student's *t*-test and simple linear regression as appropriate.

RESULTS

The biochemical data of the 20 patients (Table 1) showed total protein 7.0 \pm 0.8 g/dL, albumin 3.8 \pm 0.5 g/dL, total bilirubin 1.3 \pm 0.6 mg/dL, AST 49.5 \pm 23.4 IU/L, ALT 28.7 \pm 10.4 IU/L, and prolonged prothrombin time 1.2 \pm 0.9 s (normal < 3 s). All controls had normal blood chemistries. Gender ($P=0.11$), age ($P=0.61$), total protein ($P=0.85$), albumin ($P=0.62$), total bilirubin ($P=0.83$), ALT ($P=0.15$) and prolonged prothrombin time ($P=0.19$) were not different between those with RA < 10 mmHg and \geq 10 mmHg. Patients with RA \geq 10 mmHg had higher serum AST activities ($P=0.009$), which were related to ischemic hepatitis.

HI (3.0 \pm 0.9 L/min/m²; range: 1.6-5.3 L/min/m² vs 2.4 \pm 0.4 L/min/m²; range: 1.7-2.9 L/min/m²; $P=0.28$), AO (89.0 \pm 9.6 mmHg; range: 85-100 mmHg vs 87.3 \pm 12.8 mmHg; range: 65-115 mmHg; $P=0.78$), and LVED (12.2 \pm 6.7; range: 4-34 mmHg vs 22.1 \pm 10.9 mmHg; range: 10-40 mmHg; $P=0.06$) were not statistically different between Groups 1 and 2 (Table 2).

For all Group 1 patients, the values of PW (mean: 14.6 \pm 5.6 mmHg; range: 5-28 mmHg), PA (mean: 25.0 \pm 6.8 mmHg; range: 16-38 mmHg), RA (mean: 4.7 \pm 2.1 mmHg; range: 1-7 mmHg), and RVED (mean: 6.4 \pm 2.1 mmHg; range: 2-11 mmHg) were within the nor-

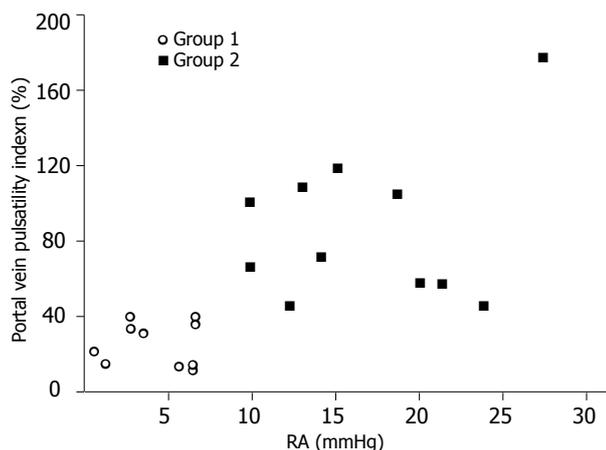


Figure 1 Portal vein pulsatility index of patients with right atrial pressure < 10 mmHg (Group 1) and ≥ 10 mmHg (Group 2).

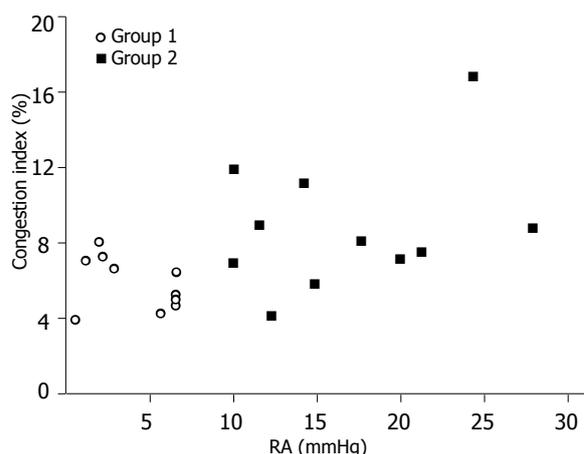


Figure 2 Congestion index of patients with right atrial pressure (RA) < 10 mmHg (Group 1) and ≥ 10 mmHg (Group 2).

Table 3 Portal profiles in patients with congestive heart failure (mean \pm SD)

| | Controls (n = 20) | RA ≤ 10 mmHg (n = 10) | RA > 10 mmHg (n = 10) |
|--------------------------|----------------------|-------------------------------|------------------------------|
| V_{\max} (cm/s) | 20.1 \pm 3.1 | 24.5 \pm 3.9 | 21.1 \pm 4.8 |
| V_{mean} (cm/s) | 11.2 \pm 1.9 | 14.0 \pm 2.3 | 12.0 \pm 2.7 |
| Area (cm ²) | 1.01 \pm 0.20 | 0.80 \pm 0.13 | 0.96 \pm 0.13 |
| PBF (mL/min) | 685 \pm 136 | 678 \pm 172 | 672 \pm 162 |
| PI (%) | 23.3 \pm 6.3 | 27.0 \pm 7.4 | 87.8 \pm 32.3 ^b |
| Congestion index | 5.3 \pm 1.2 | 5.8 \pm 1.2 | 8.7 \pm 2.4 ¹ |

RA, right atrial pressure; V_{\max} , maximum portal velocity; V_{mean} , mean portal velocity; PBF, portal blood flow; PI, portal vein pulsatility index. ^b $P < 0.001$, ¹ $P = 0.03$ vs controls and RA ≤ 10 mmHg.

mal limits.

All Group 2 patients had higher PW (mean: 29.1 \pm 7.7 mmHg; range: 13-40 mmHg; $P = 0.002$), PA (mean: 42.4 \pm 12.0 mmHg; range: 25-65 mmHg; $P = 0.004$), RA (mean: 16.8 \pm 4.9 mmHg; range: 10-28 mmHg; $P < 0.001$), and RVED (mean: 17.8 \pm 4.4 mmHg; range: 9-26 mmHg; $P < 0.001$) than Group 1 patients.

The healthy controls had V_{\max} 20.1 \pm 3.1 cm/s, V_{mean} 11.2 \pm 1.9 cm/s, area 1.01 \pm 0.20 cm², PBF 685 \pm 136 mL/min, PI 23.3 \pm 6.3%, and CI 5.3 \pm 1.2. The mean values of V_{\max} (24.5 \pm 3.9 cm/s; range 17-33 cm/s vs 21.1 \pm 4.8 cm/s; range 14-33 cm/s; $P = 0.17$), V_{mean} (14.0 \pm 2.3 cm/s; range: 9.7-18.8 cm/s vs 12.0 \pm 2.7 cm/s; range: 8.6-18.8 mmHg; $P = 0.15$) and PBF (678 \pm 172 mL/min; range: 373-1 120 mL/min vs 672 \pm 162 mL/min; range: 432-922 mL/min; $P = 0.95$) between Groups 1 and 2 did not show any statistical difference (Table 3). Group 2 patients had a larger area of portal vein than that of Group 1 (0.80 \pm 0.13 cm²; range: 0.64-1.13 cm² vs 0.96 \pm 0.13 cm²; range: 0.79-1.33 cm²; $P = 0.04$).

All the 10 patients in Group 1 had a continuous antegrade portal flow with a mean PI 27.0 \pm 7.4% (range: 17-40%) (Figure 1). The mean PI of the 10 patients in Group 2 was 87.8 \pm 32.2% (range: 43-194%). In Group 2, all the patients had a PI > 40%. Six of them had transient reduced portal blood flow, one had stagnant flow, and

three had hepatofugal flow.

Group 2 patients (mean: 8.7 \pm 2.4, range: 5.9-16.7) had a higher CI than that of Group 1 patients (mean: 5.8 \pm 1.2, range: 3.9-8.3; $P = 0.03$). Although Group 2 had a higher mean CI than Group 1, the CI could be as low as 5.9 in Group 2 and as high as 8.3 in Group 1 (Figure 2).

Using linear regression, CI showed a good correlation with PI ($P < 0.001$), PW ($P < 0.001$), PA ($P < 0.001$), RA ($P = 0.043$), RVED ($P = 0.005$), HI ($P < 0.001$), AO ($P < 0.001$), CO ($P < 0.001$), LVED ($P < 0.001$), V_{\max} ($P < 0.001$), V_{mean} ($P < 0.001$), area ($P < 0.001$) and PBF ($P < 0.001$).

DISCUSSION

It is well known that the passive "backward" congested liver develops into hepatomegaly, synchronous pulsation, engorged and dilated terminal hepatic veins, atrophy of hepatocytes and eventually cardiac cirrhosis. The high hepatic vein pressure can transmit through the liver to cause post-sinusoidal portal hypertension, cardiac ascites and change of portal vein flow patterns^[12,13]. Therefore, the changes of portal flow may help the assessment of heart function.

Prolonged right heart failure may result in atrophy of hepatocytes and eventually cardiac cirrhosis^[3]. In the present study, we have strived to exclude those patients with chronic liver disease. The abdominal sonographies showed no splenomegaly or coarse liver echogenicity and the peripheral blood showed no abnormal reduction of leukocyte, hemoglobin or platelet account, which were common in cirrhosis. Furthermore, the portal flow pattern did not show reduced fluctuation, which was common in cirrhosis with portal hypertension^[4]. Our patients were not likely to develop obvious cardiac cirrhosis.

In the present study, all patients with RA ≥ 10 mmHg had a PI > 40% and all patients with RA < 10 had a PI < 40% or less. The findings were consistent with our prior study^[10] that PI showed a good correlation with PW, PA, RA, and RVED. The waveform changes of portal blood flow correlate well with right heart function, and the measurement of PI change is a simple and non-

invasive method to identify right heart failure^[10]. Our data also demonstrated that PI had no any correlation with HI, AO, CO, LVED, V_{max} , V_{mean} and PBF. Furthermore, the waveform changes of portal blood flow correlated well with right heart function; and the PI is helpful for the diagnosis of stagnant or hepatofugal portal blood flow but not by the CI^[10]. Therefore, CI is better than PI in the assessment of left heart function.

In addition to the assessment of left heart function, the CI correlated with all PBF, V_{max} , V_{mean} , area, PI, HI, PW, PA, RA, AO, CO, LVED, and RVED. These results suggest that CI also correlates well with right heart profiles. Our findings were consistent with earlier studies^[12,20,21]. However, the CI values could be as high as 8.3 in patients with RA < 10 mmHg and as low as 5.9 in those with RA \geq 10 mmHg. If the CI value is between 5.9 and 8.3, it is difficult to predict whether or not the RA values \geq 10 mmHg. Therefore, RI is a more significant indicator than CI in the clinical evaluation of high RA \geq 10 mmHg.

The occurrence of congestive liver is not uncommon in patients with congestive heart failure. In addition to the occurrence of congestive hepatomegaly and dilatation of inferior vena cava and hepatic veins during abdominal sonography, the measurement of both CI and PI is helpful for the indirect non-invasive evaluation of cardiac function.

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RAPID COMMUNICATION

Successful laparoscopic management for cholecystoenteric fistula

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Abstract

AIM: Since 1987, laparoscopic cholecystectomy (LC) has been widely used as the favored treatment for gallbladder lesions. Cholecystoenteric fistula (CF) is an uncommon complication of the gallbladder disease, which has been one of the reasons for the conversion from LC to open cholecystectomy. Here, we have reported four cases of CF managed successfully by laparoscopic approach without conversion to open cholecystectomy.

METHODS: During the 4-year period from 2000 to 2004, the medical records of the four patients with CF treated successfully with laparoscopic management at the Chang Gung Memorial Hospital-Taipei were retrospectively reviewed.

RESULTS: The study comprised two male and two female patients with ages ranging from 36 to 74 years (median: 53.5 years). All the four patients had right upper quadrant pain. Two of the four patients were detected with pneumobilia by abdominal ultrasonography. One patient was diagnosed with cholecystocolic fistula preoperatively correctly by endoscopic retrograde cholangiopancreatography and the other one was diagnosed as cholecystoduodenal fistula by magnetic resonance cholangiopancreatography. Correct preoperative diagnosis of CF was made in two of the four patients with 50% preoperative diagnostic rate. All the four patients underwent LC and closure of the fistula was carried out by using Endo-GIA successfully with uneventful postoperative courses. The hospital stay of the four patients ranged from 7 to 10 d (median, 8 d).

CONCLUSION: CF is a known complication of chronic gallbladder disease that is traditionally considered as a contraindication to LC. Correct preoperative diagnosis of CF demands high index of suspicion and determines the success of laparoscopic management for the subset

of patients. The difficult laparoscopic repair is safe and effective in the experienced hands of laparoscopic surgeons.

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Key words: Laparoscopic management; Cholecystoenteric fistula

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INTRODUCTION

Since 1987, laparoscopic cholecystectomy (LC) has been the favored treatment for gallbladder lesions^[1]. With experience gained from laparoscopic surgery, LC has successfully been attempted in every kind of gallbladder disease.

Cholecystoenteric fistula (CF) is an uncommon disease affecting both biliary and the gastrointestinal tracts, with reported incidences ranging from 0.15% to 5% of biliary disease^[2]. Chronic cholecystitis with gallstones is the primary etiology in as many as 75% of CF patients. Cholecystoduodenal type accounts for as many as 80% of CF^[3]. CF is generally considered to be a relative contraindication to LC because of the difficulties involved in its management intraoperatively^[4]. Laparoscopic stapling techniques have been reported as feasible and safe methods for treating such fistula^[2,3,5-7]; however, these procedures are not always performed successfully. We have reported four cases of CF managed successfully by laparoscopic approach due to the correct preoperative diagnosis and experienced laparoscopic techniques.

MATERIALS AND METHODS

From January 2000 to December 2003, 4131 patients underwent LC for gallbladder lesions at the Department of General Surgery, Chang Gung Memorial Hospital (Taipei, Taiwan), 4 (0.1%) of them were identified as having CF. CF was defined as an abnormal communication between gallbladder and the neighboring organ. This study retrospectively reviewed the medical records of these four patients. All the patients' history was recorded and under-

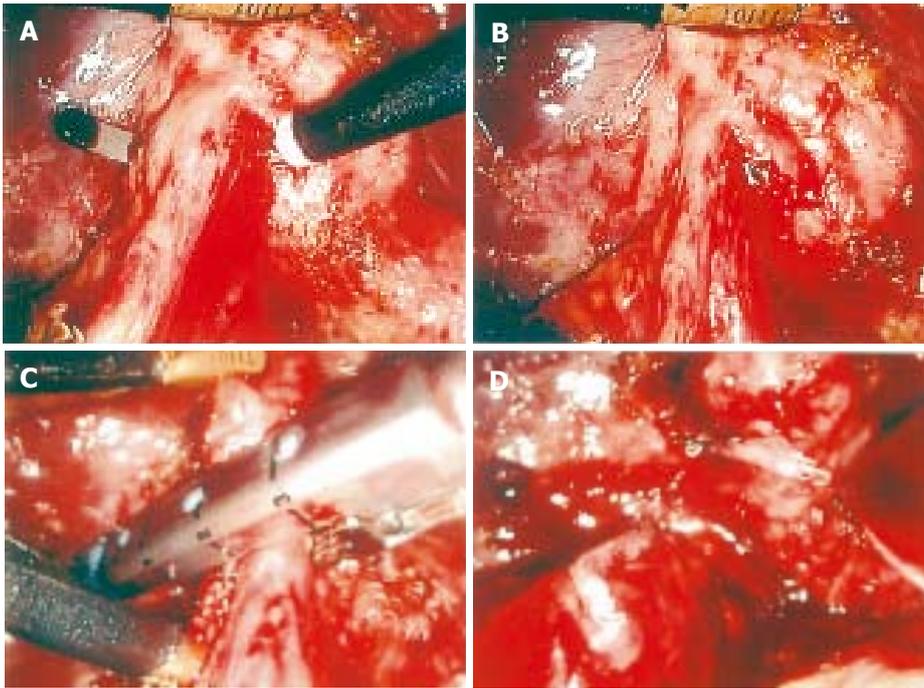


Figure 1 Procedures showing management of cholecystoenteric fistula. **A:** Identification of cholecystocolic fistula; **B:** fistula is clearly exposed; **C:** fistula tract communicating with the transverse colon is resected using an Endo-GIA device; **D:** fistula is disconnected.

went physical examination, ultrasonography (USG), or endoscopic retrograde cholangiopancreatography (ERCP), or computed tomography (CT), or magnetic resonance cholangiopancreatogram (MRCP) to establish a preoperative diagnosis. Preoperative ERCP was done routinely in patients with a history of jaundice, cholangitis or pancreatitis, patients with dilated common bile duct (CBD) displayed on abdominal USG, and patients with abnormal liver function tests. Patients with CBD stones had the stones removed endoscopically before LC. Moreover, endoscopic papillotomy (EPT) with stone retrieval and/or endonasobiliary drainage (ENBD) was performed to relieve fever, jaundice, and/or CBD stone retrieval. Data were collected on patients' age, sex, preoperative diagnoses, operative findings, operative methods, morbidity, and management.

Surgery was performed under general anesthesia using the standard four-cannula technique. The cystic artery generally was first controlled using metallic clips and divided. Moreover, the fistula was cleaned until the anatomy was clear and adequate space existed to apply an Endo-GIA (US Surgical Corp., Norwalk, CT, USA). Endo-GIA was applied via the epigastric port and then was applied to the adjacent organ end of the fistula using an applicator (Ethicon Endo-surgery). One of the blades of the Endo-GIA with the locking mechanism at its end must be seen clearly behind the fistula tract and the locking mechanism should be free of the intervening tissue to facilitate and ensure Endo-GIA locking. The gallbladder end of the fistula was ligated and simultaneously the fistula was divided (Figures 1A-1D). The gallbladder was dissected from the liver using electrocautery and removed via the umbilical or epigastric wound with an endo-bag. The postoperative course and complications were recorded. Patients were followed up in the outpatient clinic one month after the surgery and at three-monthly intervals for one year. Patients who operated more than one year before were interviewed by telephone to identify any unrecorded complications before the conclusion of the study (2004, June).

Table 1 Demographic and laboratory data of patients with cholecystoenteric fistula

| Patients | 1 | 2 | 3 | 4 |
|-----------------------|----------|-------------------------------|----------|----------|
| Age (yr) | 63 | 74 | 36 | 44 |
| Gender | F | M | M | F |
| Symptoms and signs | RUQ pain | RUQ pain, fever, and jaundice | RUQ pain | RUQ pain |
| Hemoglobin (g/dL) | 7.9 | 9.7 | 8.2 | 9.5 |
| WBC (1 000/dL) | 11.4 | 14.3 | 14.1 | 13.5 |
| Bilirubin (T) (mg/dL) | 0.8 | 8.3 | 1.3 | 0.4 |
| AST (IU/L) | 20 | 339 | 17 | 51 |
| ALT (IU/L) | 15 | 319 | 30 | 62 |
| ALP (IU/L) | 347 | 182 | 74 | 82 |

M: male; F: female; WBC: white blood cell; AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase; RUQ: right upper quadrant.

RESULTS

Age and sex

The study comprised two male and two female patients with ages ranging from 36 to 74 years (median: 53.5 years, Table 1). All the four patients exhibited right upper quadrant pain. Table 1 summarizes the laboratory data of these four patients. All the four patients revealed anemia and leukocytosis. Only one patient revealed elevated bilirubin level due to common bile duct stone and cholangitis.

Preoperative investigations, diagnoses, managements, and operative outcomes

Table 2 displays the preoperative investigations, diagnoses, managements, and operative outcomes. Two of the four patients were detected as gallbladder stone with pneumobilia by abdominal USG. One patient had gallbladder stone and a cholecystocolic fistula displayed by ERCP (patient 1,

Table 2 Preoperative investigations, diagnoses, managements, and operative outcomes of patients with cholecystoenteric fistula

| Patients | 1 | 2 | 3 | 4 |
|--------------------------|--|--|--|--|
| USG | GB stone, pneumobilia, and CBD stone | GB stone and biliary tree dilatation | Contracted GB with stone and biliary tree dilatation | GB stone and pneumobilia |
| ERCP/EPT/stone retrieval | +(fistula)/+/+ | +(pneumobilia)/+/+ | +/-/- | -/-/- |
| CT/MRCP | -/- | -/- | -/- | -/+ (fistula) |
| Preoperative diagnosis | Cholecystocolic fistula | GB stone and CBD stone | Cystic duct stone and normal bile duct | Cholecystoduodenal fistula |
| Associate biliary lesion | GB stone and CBD stone | GB stone and CBD stone | GB stone | GB stone |
| Operative findings | Cholecystocolic fistula | Cholecystoduodenal fistula | Cholecystocholedochal fistula (Mirizzi syndrome type II) | Cholecystoduodenal fistula |
| Operative method | LC and closure of fistula using Endo-GIA | LC and closure of fistula using Endo-GIA | LC and closure of fistula using Endo-GIA | LC and closure of fistula using Endo-GIA |
| Cause of conversion | Nil | Nil | Nil | Nil |
| Morbidity | Nil | Nil | Nil | Nil |
| Hospital stay (d) | 8 | 8 | 7 | 10 |

GB: gallbladder; CBD: common bile duct; ERCP: endoscopic retrograde cholangiopancreatography; EPT: endoscopic papillotomy; MRCP: magnetic resonance cholangiopancreatography; CT: computed tomography; USG: ultrasonography.

Figure 2A). ERCP revealed opacification of the gallbladder and abnormal fistula communication with colon demonstrated by haustration. The other one had pneumobilia revealed by ERCP (patient 2, Figure 2B). MRCP diagnosed cholecystoduodenal fistula in one patient as demonstrated by air-fluid level and abnormal communication between gallbladder and duodenum in T2-weighted image (patient 4, Figures 2C and 2D). Correct preoperative diagnosis of CF was made in two of the four patients with 50% preoperative diagnostic rate. Operative findings revealed two cholecystoduodenal fistula (patients 2 and 4), one cholecystocolic fistula (patient 1), and one cholecystocholedochal fistula (Mirizzi syndrome type II, patient 3). All the four patients underwent LC and closure of the fistula by Endo-GIA (after successful identification of the fistula). None of the patients needed to be converted to open cholecystectomy and all the four patients had uneventful postoperative course. None of the CF was caused by malignancy. The hospital stay of the four patients ranged from 7 to 10 d (median, 8 d). Length of follow-up ranged from 8 to 48 mo (median, 36.5 mo).

DISCUSSION

As we know, the development of a fistulous tract from the gallbladder is associated with gallstones in 90% of cases. Our study demonstrated CF was associated with gallstones in 100% cases. As seen in this study, patients with a biliary-enteric fistula are commonly presented with signs and symptoms similar to those of chronic cholecystitis. These lack specific symptoms that suggest the presence or development of a biliary-intestinal fistula. Right upper quadrant pain was present in all the CF patients, irrespective of the level of the fistula. Yamashita *et al*^[8] reviewed 33 (1.3%) of 1 929 consecutive patients who had been treated for biliary tract diseases during a 12-year period had biliary fistulas. In this study, we found that the incidence of CF was only

0.1%, showing a decreased incidence these years in Asia corresponding to Western observations. CF is generally considered to be a relative contraindication to LC because of the difficulties involved in its management intraoperatively, and CF is therefore usually managed by laparotomy. This may partly explain the reason of lower incidence in this series.

Preoperative findings can be suggestive of internal biliary fistula. Pneumobilia with an atrophic gallbladder adherent to neighboring organs seen on CT scanning or USG is highly suggestive of the presence of cholecystoenteric fistula, as seen in patients 1 and 4^[4]. However, the reliability of a diagnosis based on the presence or absence of pneumobilia has been questioned. Similar to the report by Yamashita *et al*^[8], ERCP was the most valuable diagnostic method for revealing the presence of biliary-enteric fistula. As seen in the patient 1, ERCP could be used to diagnose the cholecystocolic fistula by demonstration of the haustration of the colon. In this study, we were able to diagnose biliary-enteric fistula preoperatively by USG, ERCP, or MRCP, and could therefore decide on a treatment strategy preoperatively. Besides, MRCP provides another reliable diagnostic tool to clearly demonstrate the abnormal communication, especially in T2-weighted image. However, only 50% of CF was diagnosed preoperatively in our series.

Treatment advocated for CF is cholecystectomy and closure of the fistula communication. Making a knot either intracorporeally or extracorporeally is time-consuming and cannot secure this difficult fistula closure. An alternative is applying laparoscopic intracorporeal suturing interruptedly or continuously with absorbable or non-absorbable material to close the inflammatory fistula, as performed in the open procedure. However, this method is technically demanding and time-consuming^[5]. Ligation of the fistula with an endoloop offers a solution to the problem. The technique for the application of an endoloop consists

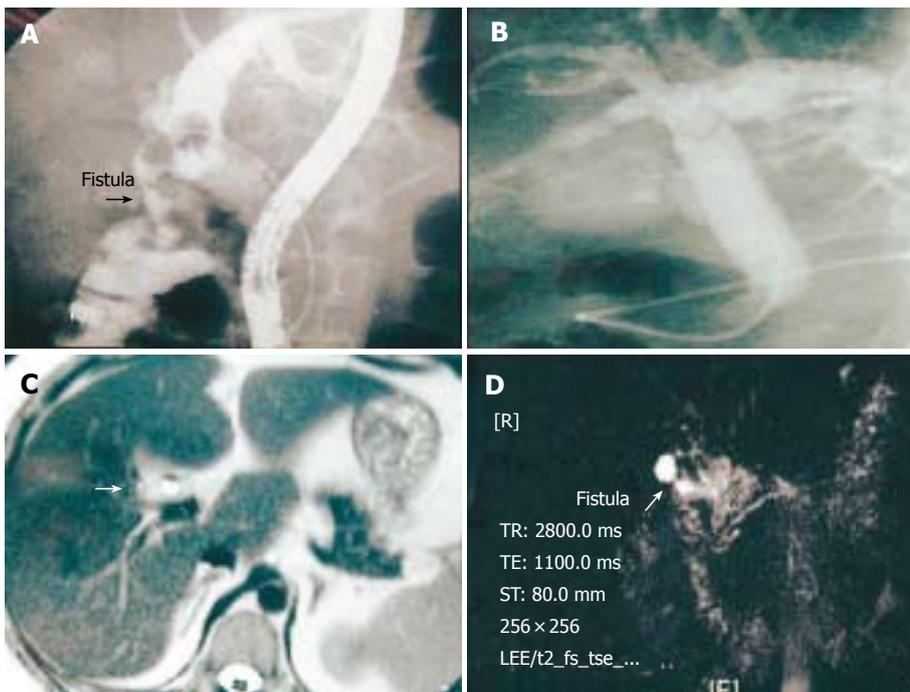


Figure 2 Detection of cholecystoenteric fistula radiologically. **A:** ERCP showing appearance of pneumobilia with common bile duct stone and a fistula between collapsed gallbladder and transverse colon; **B:** ERCP showing common bile duct stone and pneumobilia; **C:** MRCP T2-weighted image showing pneumobilia and air-fluid level within the gallbladder; **D:** MRCP T2-weighted image revealing fistula between collapsed gallbladder and duodenum.

of dividing the fistula and then applying the endoloop. This becomes more difficult if, after the fistula is divided, loss of traction on the communicating organ results in retraction of the divided fistula stump outside of the laparoscopic field of view. Although endoloop is cheaper than Endo-GIA, some new devices are needed to apply to avoid this difficulty^[9]. Endo-GIA is easy to use and effective. However, the following important safety points must be noted. First, the fistula must be cleaned until the anatomy of the abnormal communication is clear and adequate space exists to apply an Endo-GIA. Second, one of the blades of the Endo-GIA with the locking mechanism at its end must be seen clearly behind the communicating organ and the locking mechanism should be free of the intervening tissue to ensure successful Endo-GIA locking. The above ensures safe closure of the fistula tract with the communicating organ. Especially, when resecting the fistula laparoscopically, it is important to ensure that part of the colonic wall is included in the excised specimen. Two reasons explain the maneuver, including that histological examination is essential to rule out colonic carcinoma at the site of fistula and unhealthy fistula or gallbladder tissue, if left on the wall of the colon, can become ischemic at a later date, thereby subsequently leading to the perforation of the wall^[6]. However, no malignancy was seen in the colonic wall of the cholecystocolonic fistula in this study.

In conclusion, this report shows that with increasing experience and confidence, contraindications to laparoscopic cholecystectomy are diminishing. Laparoscopic management of CF with Endo-GIA is feasible and safe, if

CF is diagnosed correctly preoperatively and managed by experienced laparoscopic surgeons.

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RAPID COMMUNICATION

Interleukin-1 β gene polymorphism associated with hepatocellular carcinoma in hepatitis B virus infection

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hepatitis B patients in Thai population.

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Abstract

AIM: To examine the effect of interleukin-1-beta (IL-1 β) promoter region C-511T and IL-1 receptor antagonist (IL-1RN) polymorphism among the patients with chronic hepatitis B virus (HBV) infection (HCC and non-HCC).

METHODS: Genomic DNA from 136 Thai patients with chronic HBV infection (HCC = 46 and non-HCC = 90) and 152 healthy individuals was genotyped for IL-1 β gene polymorphism (-511) using polymerase chain reaction with sequence specific primers (PCR-SSP). The variable number of tandem repeats (VNTR) of IL-1RN gene was assessed by a PCR-based assay. The association between these genes and status of the disease was evaluated by χ^2 test.

RESULTS: IL-1B-511 genotype C/C was found to be significantly different in patients with HCC when compared with healthy individuals ($P = 0.036$, OR = 2.29, 95%CI = 1.05-4.97) and patients without HCC ($P = 0.036$, OR = 2.52, 95%CI = 1.05-6.04). Analysis of allele frequencies of IL-1B-511 showed that IL-1B-511 C allele was also significantly increased in patients with HCC, compared to that in healthy control ($P = 0.033$, OR = 1.72, 95%CI = 1.04-2.84). However, no significant association in IL-1RN gene was found between the two groups.

CONCLUSION: IL-1B-511C allele, which may be associated with high IL-1B production in the liver, is a genetic marker for the development of HCC in chronic

INTRODUCTION

Hepatitis B virus (HBV) is the most common cause of acute and chronic liver disease worldwide, especially in Asia and Africa. It has been estimated that more than 350 million people worldwide, representing more than 5% of world population are carriers of HBV infection^[1,2]. Approximately 250 000 deaths occur each year resulting from fulminant hepatic failure, cirrhosis, and hepatocellular carcinoma (HCC)^[3]. Family studies in China provide some evidence that the host genetic factors influence viral persistence, as a higher concordance rate has been found for HBeAg persistence in identical twins compared with non-identical twins^[4]. Thus, it is conceivable that genetic difference may affect the different outcomes of patients with HBV infection. Several genetic studies on the hosts have reported that human leukocyte antigen (HLA) genes^[5-7] and various cytokine genes (TNF- α , IFN- γ , IL-12, IL-18, TGF- β , IL-10, IL-4) are associated with HBV susceptibility and/or HBV persistence or disease severity^[8-11].

Interleukin-1 (IL-1) is a proinflammatory cytokine with multiple biological effects^[12]. The IL-1 gene family (including IL-1A, IL-1B, and IL-1RN) on chromosome 2q13-21 encodes three proteins, which comprise the agonists IL-1 α , IL-1 β , and their naturally occurring inhibitor, IL-1 receptor antagonist (IL-1RN)^[12,13]. Recently, allele 2 of IL-1RN intron 2 has been reported as a resistant marker of HBV infection, suggesting the role of IL-1 polymorphisms in the pathogenesis of developing chronic hepatitis B^[14]. This allele is associated with enhanced IL-1 β production *in vitro*^[15] and *in vivo*^[16]. Therefore, Zhang *et al.*^[14] hypothesized that high production of IL-1 β may help

increase the production of other cytokines such as IL-2, IL-6, and TNF- α and trigger the complex immunological processes to eliminate the virus. Interestingly, besides its major role as a proinflammatory cytokine, IL-1 β has been implicated as an important factor for tumor growth^[17-19]. Several independent lines of evidence have also suggested that genetic polymorphisms within IL-1 β gene are associated with gastric cancer and HCC in HCV infection^[20-22].

The aim of the present study was to determine the genotype and allele frequencies of IL-1B-511 and IL-1RN VNTR polymorphisms among the Thai patients with chronic HBV infection (HCC and non-HCC) and healthy individuals to assess whether these genes are involved in chronic HBV susceptibility and/or HCC development.

MATERIALS AND METHODS

Subjects

One hundred and thirty-six Thai patients with chronic HBV infection were recruited into this study from Chulalongkorn Memorial Hospital. The diagnosis of chronic hepatitis B was established by seropositivity for HBsAg over a 6-month period and did not have any other type of liver diseases such as chronic hepatitis C or alcoholic liver disease. In addition, all the patients had elevated serum ALT and AST levels. Patients with chronic HBV infection were further divided into two groups: without ($n=90$) and with HCC ($n=46$) according to the absence or presence of concurrent HCC. Diagnosis of HCC was based on histopathology and/or a combination of mass lesion in the liver from hepatic imaging and serum alpha fetoprotein level >400 ng/mL. Moreover, 152 ethnically and geographically matched controls from healthy blood donors of the Thai Red Cross Society were recruited.

Genotyping for SNPs of IL-1B and IL-1RN genes

Molecular genetic analysis was performed on genomic DNA obtained from peripheral blood leukocytes using standard salting-out method as previously described^[23]. SNP at position -511 of IL-1B was genotyped by polymerase chain reaction (PCR) with sequence specific primer (PCR-SSP) (F-5' CTCATCTGGCATTGATCTGG-3' and R-5' GGTGCTGTTCTCTGCCTCGA-3')^[24]. The PCR conditions were established as previously described^[25].

The VNTR of IL-1RN gene was assessed by a PCR-based assay. Oligonucleotides F-5' CTCAGCAACACTCCTAT-3' and R-5' TCCTGGTCTGCAGGTAA-3' flanking this region were used as primers^[24]. The PCR conditions were an initial denaturation at 94 °C for 2 min, followed by 35 cycles at 94 °C for 20 s, at 59 °C for 50 s, at 72 °C for 20 s, and a final extension at 72 °C for 7 min. Each allele was identified according to its size^[24].

Statistical analysis

The association between these genes and disease status was evaluated by the statcalc from Epi info version 6 program^[26] to calculate the odds ratio (OR) and 95% confidence interval (CI), Yates' corrected χ^2 and associated

Table 1 IL-1 β and IL-1RN polymorphisms in patients with HBV and healthy controls

| Polymorphisms | Patients with HBV | | Healthy controls $n = 152$ (%) |
|----------------------|-----------------------------|---------------------------|-----------------------------------|
| | Without HCC $n = 90$ (%) | With HCC $n = 46$ (%) | |
| IL-1B-511 | | | |
| Genotype frequencies | | | |
| C/C | 17 (18.89) | 17 (36.96) ^{1,2} | 31 (20.39) |
| C/T | 51 (56.67) | 21 (45.65) | 79 (51.97) |
| T/T | 22 (24.44) | 8 (17.39) | 42 (27.63) |
| Allele frequencies | | | |
| C | 85 (47.22) | 55 (59.78) ³ | 141 (46.38) |
| T | 95 (52.78) | 37 (40.22) | 163 (53.62) |
| IL-1RN | | | |
| Genotype frequencies | | | |
| 1/1 | 74 (82.22) | 38 (82.61) | 121 (79.61) |
| 1/2 | 15 (16.67) | 8 (17.39) | 29 (19.08) |
| 2/2 | 1 (1.11) | 0 | 1 (0.66) |
| 1/4 | 0 | 0 | 1 (0.66) |
| Allele frequencies | | | |
| 1 | 163 (90.56) | 84 (91.30) | 272 (89.47) |
| 2 | 17 (9.44) | 8 (8.70) | 31 (10.20) |
| 4 | 0 | 0 | 1 (0.33) |

¹ $P=0.036$ vs healthy controls, OR (95%CI)=2.29 (1.05-4.97); ² $P=0.036$ vs patients without HCC, OR (95%CI)=2.52 (1.05-6.04); ³ $P=0.033$ vs healthy controls, OR (95%CI)=1.72 (1.04-2.84).

P values. $P < 0.05$ was considered statistically significant. The groups were tested for conformity to the Hardy-Weinberg equilibrium by $2 \times 2 \chi^2$ test comparing observed and expected numbers.

RESULTS

The genotype and allele frequencies of IL-1B and IL-1RN in healthy control subjects and patients with chronic hepatitis B, including patients with and without HCC are shown in Table 1. All the three groups were in Hardy-Weinberg equilibrium with no significant χ^2 values compared to the observed and expected genotype frequencies of each of the tested polymorphisms. The heterozygous C/T of IL-1B was the most common genotype in all the three groups (51.97% in healthy controls, 45.65% in patients with HCC and 56.67% in patients without HCC). The homozygous T/T was the second most common genotype in healthy controls (27.63%) and patients without HCC (24.44%), followed by the C/C genotype that was found in 20.39% of healthy controls and in 18.89% of patients without HCC, respectively. In contrast to these two groups, the C/C was the second most common genotype in patients with HCC (36.96%), followed by the T/T genotype (17.39%). Comparison of IL-1B-511 genotype revealed that IL-1B-511 C/C genotype was significantly increased in patients with HCC compared to that in healthy controls ($P=0.036$, OR = 2.29, 95%CI = 1.05-4.97) and patients without HCC ($P=0.036$, OR = 2.52, 95%CI = 1.05-6.04). Analysis of allele frequencies of IL-1B-511 showed

that IL-1B-511 C allele was also significantly increased in patients with HCC, compared to that in the healthy controls ($P=0.033$, $OR=1.72$, $95\%CI=1.04-2.84$). The effect of IL-1B-511 C allele was similar to autosomal recessive mode of inheritance. The presence of two C alleles (CC) was required to increase the likelihood of HCC development.

Four genotypes of IL-1RN (1/1, 1/2, 2/2, 1/4) were found in this study. The IL-1RN 1/1 genotype was the most common genotype in all the three groups (79.61% in healthy controls, 82.61% in patients with HCC and 82.22% in patients without HCC), followed by 1/2 genotype that was 19.08% in healthy controls, 17.39% in patients with HCC and 16.67% in patients without HCC, respectively. The 2/2 genotype was 0.66% in healthy control and 1.11% in patients without HCC, whereas the 1/4 genotype was 0.66% in healthy controls. There were no significant differences in genotype or allele frequencies of IL-1RN between patients with chronic hepatitis B and healthy controls.

DISCUSSION

The association between the development of chronic hepatitis B and the polymorphisms of IL-1B gene (-511C/T) and IL-1RN gene (VNTR at intron 2) was not observed in this study. However, the -511C allele of IL-1B gene was identified as a genetic marker for the development of HCC in patients with chronic HBV infection. The hypothesis regarding IL-1 genetic polymorphism and hepatocarcinogenesis is based on the assumption that carriers of these genotypes are associated with increased levels of IL-1B in the liver in response to HBV infection and hepatocyte damage that may finally lead to the development of HCC. This hypothesis is supported by the observation that IL-1B level is increased in the liver tissue surrounding the tumor tissue^[27]. IL-1B is a proinflammatory cytokine as well as a tumor growth factor. There are several lines of evidence that support its role in tumor growth development. First, IL-1B can increase the production of prostaglandin E2 and hepatocyte growth factor^[17]. Second, IL-1B can induce angiogenesis which is an important step in promoting tumor growth by either upregulating COX-2 or inducing nitric oxide^[19] and vascular endothelial growth factor (VEGF)^[28]. Third, IL-1B can also attenuate interferon-induced antiviral activity and STAT1 activation in the liver^[29].

However, the IL-1B polymorphism in the promoter area in association with cancer remains controversial. Briefly, a number of studies mostly in the Caucasian population support that -511T in linkage disequilibrium with -31C is a risk haplotype for the development of gastric cancer^[30-34]. There are more conflicting data regarding the effect of IL-1B-511/-31 haplotype on the risk of gastric cancer and HCV-related HCC in Asian population, while some studies support the result from the Caucasian group^[16,21,35] and a number of studies reported that -511C/-31T is a risk haplotype for the development of cancer^[22,36-40]. Interestingly, functional studies of IL-1B genotype seem to support that -511C/-31T haplotype is associated with high-production of IL-1B. First, the IL-

1B -31 polymorphism involves a TATA sequence in the promoter and the -31T allele is associated with a five-fold elevated binding activity with the transcription initiation factor^[30,31]. Second, mucosal IL-1B level is higher than IL-1B-31T level in *H pylori*-infected gastric cancer patients^[40]. Although IL-1B -511T/-31C is associated with high level of IL-1B in the plasma^[41], it is likely that gene expression in each organ is differently regulated and the assessment of IL-1B level in targeted organ is more reliable. The functional study in liver tissue is also required for the better understanding of the role of IL-1B genotype in chronic hepatitis and HCC development.

In conclusion, IL-1B-511C allele which may be associated with the high IL-1B production in the liver is a genetic marker for the development of HCC in chronic hepatitis B patients in Thai population.

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RAPID COMMUNICATION

Increased heat shock protein 70 expression in the pancreas of rats with endotoxic shock

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Abstract

AIM: To investigate the ultra-structural changes and heat shock protein 70 (HSP70) expression in the pancreas of rats with endotoxic shock and to detect their possible relationship.

METHODS: A total of 33 Wistar rats were randomly divided into three groups: control group (given normal saline), small dose lipopolysaccharide (LPS) group (given LPS 5 mg/kg) and large dose LPS group (given LPS 10 mg/kg). Pancreas was explanted to detect the ultra-structural changes by TEM and the HSP70 expression by immunohistochemistry and Western blot.

RESULTS: Rats given small doses of LPS showed swelling and loss of mitochondrial cristae of acinar cells and increased number of autophagic vacuoles in the cytoplasm of acinar cells. Rats given large doses of LPS showed swelling, vacuolization, and obvious myeloid changes of mitochondrial cristae of acinar cells, increased number of autophagic vacuoles in the cytoplasm of acinar cells. HSP70 expression was increased compared to the control group ($P < 0.05$).

CONCLUSION: Small doses of LPS may induce stronger expression of HSP70, promote autophagocytosis and ameliorate ultra-structural injuries.

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Key words: HSP70; Pancreas; Lipopolysaccharide; Ultra-

structural changes

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INTRODUCTION

Septic shock is a clinical syndrome resulting from the systemic response of the body to infection. Septic shock is characterized by hypoperfusion of major organs, leading to multiple organ failure and finally to death. Though advances have been achieved in supportive and anti-microbial treatment, septic shock continues to be a common cause of intensive care with the mortality rate being approximately 40%^[1]. Sepsis is associated with secretory pancreatic dysfunction^[2].

Heat shock proteins (HSPs) are highly conserved molecules present in both prokaryotic and eukaryotic cells and play an important role in cellular function and stress conditions. HSPs function as molecular chaperones and are categorized into HSP90, HSP70, HSP60, HSP20, and HSP8.5 on the basis of their molecular weight. HSP70 proteins constitute the central part of the chaperone system. In mammals, two isoforms of HSP70 exist in cytoplasm: a 73-ku constitutively expressed form (HSC70) and a 72-ku stress-inducible form (HSP70). HSPs are usually constitutively present at low concentrations in cells, but are actively synthesized to reach much higher concentrations as cells react to aggressive situations^[3].

This study was to investigate the effect of different doses of LPS on the expression of HSP70 in a model of LPS-induced septic shock.

MATERIALS AND METHODS

Experimental animals

A total of 33 Wistar rats weighing 250 ± 10 g were supplied by China Medical University Animal Department. All animals received humane care.

Induction of sepsis

A total of 33 Wistar rats were randomly divided into three groups. Small dose LPS group ($n = 12$) was given a single dose of 5 mg/kg LPS, large dose LPS group ($n = 12$) was

Table 1 Expression of HSP70 in the pancreas of the three groups

| Groups | <i>n</i> | - | + | ++ | +++ |
|---------------|----------|---|---|----|-----|
| LPS5 mg/kg | 12 | 2 | 0 | 3 | 7 |
| LPS10 mg/kg | 12 | 4 | 1 | 3 | 4 |
| Control group | 9 | 2 | 6 | 1 | 0 |

given 10 mg/kg LPS, control group ($n=9$) was given the same volume of 0.9% normal saline. All rats were anesthetized with sodium pentobarbital (36 mg/kg). LPS (SIGMA, O₁₂₇B₈) was given via the femoral vein. A catheter was connected to the femoral artery to measure the mean arterial pressure. Death occurred after 3.5 h. The rats that survived were killed at 6 h. Pancreas were removed immediately and stored at -70 °C for later use.

Immunohistochemistry

Pancreatic samples were fixed in neutral buffered formalin, embedded in paraffin, sectioned, and mounted on slides. After being deparaffinized, endogenous peroxidase activity was quenched with 10% H₂O₂ for 10 min. After being blocked with goat serum, the sections were incubated with anti-mouse IgG polyclonal HSP70 antibody at 37 °C for 90 min, followed by incubation with S-A/HRP at 37 °C for 20 min. The Ag-Ab complex was visualized using diaminobenzidine method. Slides were then counterstained with hematoxylin for 1 min. After dehydration, the slides were coverslipped. To ensure specificity of the immunoreactions, control sections were subjected to the same immunohistochemical method with the exception that the primary Ab antibody was replaced with PBS.

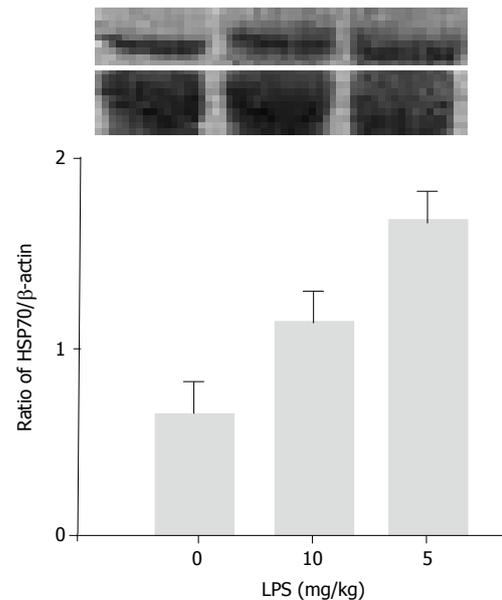
Electromicroscopic study

Pancreatic tissue was cut into 1 mm × 1 mm × 1 mm pieces and placed in a freshly prepared fixative buffer (2.5% glutaraldehyde) at 4 °C for 2 h. After being fixed in 1% osmium tetroxide at 4 °C for 1 h, the tissue was exposed twice to an increased concentration of ethanol (30%, 50%, 70%, and 99.5%) at 5-min intervals for partial dehydration. Tissue was embedded with resin in gelatin capsules and incubated at 55-60 °C for 24 h. Ultra-thin (70 nm) sections were cut and transferred to 300-mesh nickel grids. After being stained with tannic acid, saturated uranyl acetate in 50% alcohol and 0.01% lead citrate, the samples were examined under transmission electron microscope (JEM-1200EX, Tokyo, Japan).

Western blot

Tissues were sonicated on ice for approximately 10 min until the tissues were completely homogenized in a solution containing 2 mmol/L EDTA, 2 mmol/L EGTA, 0.2 mmol/L NaCl, 20 mmol/L Tris-HCl, 1% Triton, 1 mmol/L DTT, and 1 mmol/L aprotinin. The homogenate was then centrifuged at 12000 *g* for 60 min at 4 °C and the resulting supernatant fraction was transferred to a fresh tube.

An equal amount (30 µg) of protein extracts was loaded and separated by 8% SDS-polyacrylamide gel electropho-

**Figure 1** HSP70 expression in three groups.

resis (PAGE). After electrophoresis, the proteins on the gel were transferred to polyvinylidene difluoride (PVDF) membrane. After being blocked with 5% nonfat dry milk at 4 °C overnight, the PVDF membranes were incubated with anti-rabbit polyclonal antibody of HSP70 diluted 1:200 with TPBS at 37 °C for 2 h. Then the blots were reacted with goat anti-rabbit antibody at room temperature for 2 h. Pre-stained molecular mass was used to estimate the positions of various proteins on the gel. Blots were stripped and incubated with a monoclonal Ab against β-actin (Sigma) to confirm that an equal protein was loaded. The target was then detected by enhanced chemiluminescence and exposed to X-ray film for appropriate time. The pictures were analyzed by GIS TANON.

Statistical analysis

All data were expressed as mean ± SD. $P < 0.05$ was considered statistically significant.

RESULTS

TEM changes

No obvious ultra-structural damage was found in the control group. Swelling and loss of mitochondrial cristae of acinar cells and increased number of autophagic vacuoles in the cytoplasm of acinar cells were found in small dose LPS group. Swelling, vacuolization, and obvious myeloid changes of mitochondrial cristae of acinar cells and increased number of autophagic vacuoles in the cytoplasm of acinar cells were found in large dose LPS group (figure 2).

Immunohistochemistry changes

HSP70 was expressed at a low level in pancreatic acinar cells of the control group. After injection of LPS, HSP70 was highly expressed in the cytoplasm and nuclei of acinar cells and endothelia of blood vessels. Immunohistochemical examination showed overexpression

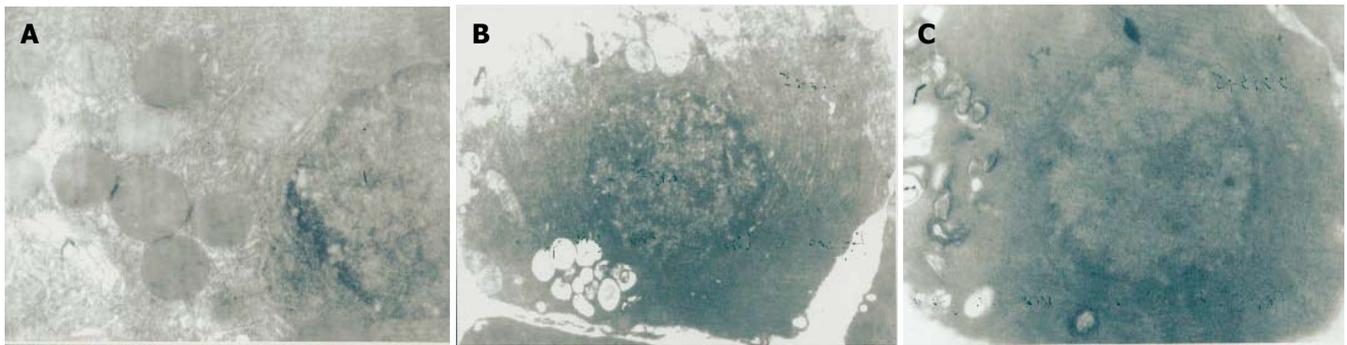


Figure 2 Ultra-structural changes of the pancreas in control (A), small dose LPS (B) and large dose LPS (C) groups.

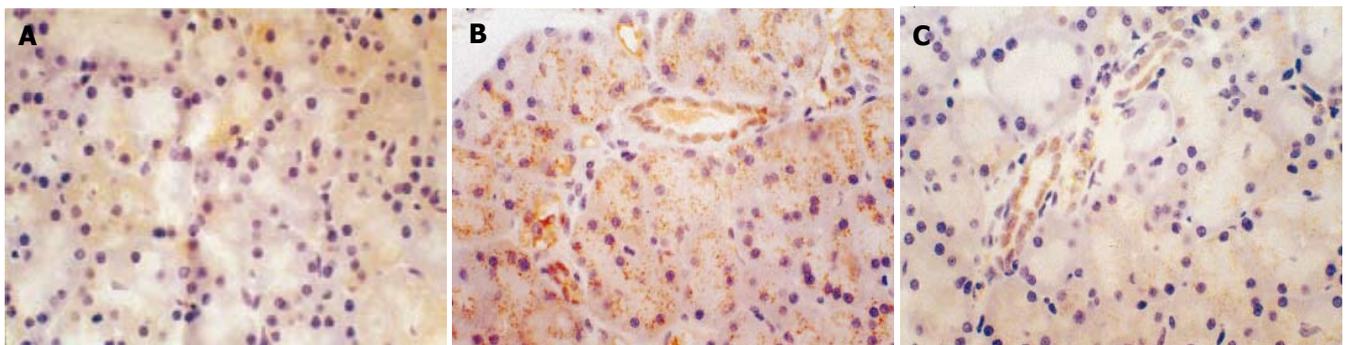


Figure 3 HSP70 expression in the pancreatic tissue of control (A), small dose LPS (B) and large dose LPS (C) groups.

of HSP70 in the vascular endothelial cells and cytoplasm of pancreatic tissue of the small dose LPS group. There were no significant differences between the two groups (Table 1).

Western blots

HSP70 expression in the control group was 112 ± 18.9 (0.62 ± 0.04). HSP70 expression in the large dose LPS group was 169 ± 13.4 (0.974 ± 0.04). HSP70 expression in the small dose LPS group was 211 ± 11.5 (1.34 ± 0.17) (Figure 1 and 3). β -Actin expression was constant, indicating the equal protein loading on the blots. HSP70 expression increased compared to the control group ($P < 0.05$).

DISCUSSION

This study showed that LPS could induce HSP70 expression in the pancreas of rats with endotoxic shock and the upregulation of HSP70 was associated with the doses of LPS and the extent of pancreatic ultra-structural injury. Endotoxin caused mitochondrial changes in the acinar cells of the pancreas and large doses of endotoxin led to myeloid changes of mitochondria. Small doses of LPS induced obvious overexpression of HSP70 and autophagocytosis, suggesting that high level of HSP70 promotes degradation of damaged proteins and ameliorates ultra-structural damages.

HSPs can protect cells from injury induced by environmental challenges, such as hypoxia, ischemia, high temperature, endotoxin, infection, and fever^[4]. The protective effects of HSPs have been confirmed in various animal models of sepsis^[5]. Several studies have demonstrated that

a preceding HSP reaction reduces both mortality and organ dysfunction in experimentally induced severe sepsis^[6]. Induction of HSPs in response to stress correlates with increased resistance to subsequent cellular damage. HSP70 is believed to act in a chaperone-like manner aiding in the passage of proteins across membrane barriers, preventing misfolding of newly synthesized proteins, facilitating elimination of improperly assembled, misfolded or aggregated proteins^[7]. HSP70 contributes to the delivery of dysfunctional proteins to lysosomes for proteolytic degradation^[8]. HSP70 is also known to facilitate antigen presentation in cells such as macrophages and dendrites^[9]. Elevations in intracellular HSP levels have been shown to improve cell tolerance to inflammatory cytokines, such as TNF- α and IL-1 β ^[10]. TNF- α and IL-1 β are thought to be the main mediators in the pathogenesis of septic shock^[11]. HSP70 expression protects the lung against ventilator-induced lung injury by decreasing cytokine transcription^[12].

Mitochondrial oxidative phosphorylation is responsible for over 90% of total body oxygen consumption and ATP generation. HSP70 upregulation protects mitochondrial function after ischemia-reperfusion injury^[13]. Increased levels of HSP70 through gene transfection lead to a greater protection of mitochondrial function after ischemia-reperfusion in a donor heart preservation protocol^[13]. This may be one mechanism by which HSP70 overexpression leads to a better protection. Overexpression of HSP70 in the whole pancreas can protect mitochondrial integrity^[14]. It was reported that structural deformity and decrease of respiratory chain enzyme activity in mitochondria and decline of ATP content are highly correlated with the deterioration of cardiac function during sepsis^[15]. Heat shock

pretreatment prevents cardiac mitochondrial dysfunction during sepsis^[16], thus achieving a protective goal.

HSP70 protection against LPS is most probably mediated through the modulation of iNOS activation and the subsequent decreased synthesis of nitric oxide (NO)^[17]. NO plays an important role in the pathogenesis of septic shock^[18]. A key role of HSP70 in the natural resistance of human beta cells against NO-induced injury is by preserving mitochondrial function^[19]. Constitutive expression of HSP70 in human beta cells is essential for the natural resistance against NO-induced injury^[19]. HSP70 prevents secretagogue-induced cell injury in the pancreas^[20]. Hyperthermia protects against arginine-induced pancreatitis and induces HSP70 in the pancreas^[21].

After entering blood, endotoxin combines with CD14^[22]. Both CD14 and TLR2 are receptors of LPS^[22]. TLR-4 and CD14 are involved in HSP70-mediated proinflammatory responses^[23] and in HSP70-mediated activation of innate immunity^[24]. Further investigation is necessary to elucidate the mechanism underlying the induction of endotoxin by HSP70.

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RAPID COMMUNICATION

Interferon alpha plus ribavirin combination treatment of Japanese chronic hepatitis C patients with HCV genotype 2: A project of the Kyushu University Liver Disease Study Group

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wk after the end of treatment, was remarkably high by 84.4%, (146/173) by an intention-to-treat analysis. A significant difference in SVR was found between patients with and without the discontinuation of ribavirin (46.9% vs 92.9%), but no difference was found between those with and without a dose reduction of ribavirin. A significant difference in SVR was also found between patients with less than 16 wk and patients with 16 or more weeks of ribavirin treatment (34.8% vs 92.0%).

CONCLUSION: The 24-wk interferon and ribavirin treatment is highly effective for Japanese patients with HCV genotype 2. The significant predictor of SVR is continuation of the ribavirin treatment for up to 16 weeks.

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Key words: Hepatitis C virus; Interferon; Ribavirin; Genotype 2

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Abstract

AIM: To determine the efficacy of an interferon alpha and ribavirin combination treatment for Japanese patients infected with hepatitis C virus (HCV) of genotype 2, a multi-center study was retrospectively analyzed.

METHODS: In total, 173 patients with HCV genotype 2 started to receive interferon-alpha subcutaneously thrice a week and 600–800 mg of ribavirin daily for 24 wk.

RESULTS: The overall sustained virological response (SVR), defined as undetectable HCV RNA in serum, 24

INTRODUCTION

The heterogeneity of the hepatitis C virus (HCV) genome has warranted the classification of the virus into different genotypes, with six major genotypes and more than 50 subtypes of HCV having been described till date^[1-3]. The different genotypes may be important to the pathogenesis of the disease^[4], response to antiviral therapy^[5], and the diagnosis^[6], as shown by molecular epidemiological studies and research on vaccine development.

A currently popular treatment regimen for the treat-

ment of chronic HCV infection in the world is pegylated interferon (IFN) alpha in combination with ribavirin. However, there was no data of response to such combination treatment for Japanese patients, because the treatment was just approved by the Japanese Minister of Health, Labour and Welfare in December 2004. Treatment with these drugs has resulted in a high rate of sustained virological response (SVR), over 50%^[7,8]; however, the treatment duration is long, 48 wk and it causes various side effects, which are sometimes serious. Such a combination treatment is also expensive; a 24-wk treatment course costs approximately \$20 000^[9]. The efficacy and economic aspects need to be analyzed. Quite recently, a very short duration treatment for acute hepatitis C was shown to be highly effective^[10].

The HCV genotype has been reported to be the most important predictor of IFN treatment response^[7-13]. Patients infected with genotypes 2 and 3 have achieved about 65% SVR in a trial of 24-wk IFN alpha in combination with ribavirin, in contrast to patients with genotype 1 who had under 30% SVR^[14,15]. Recently, multicenter studies in Europe and North America showed that patients with genotypes 2 and 3 were able to achieve a high SVR in a trial of 14-16 wk of pegylated IFN alpha in combination with ribavirin^[16,17]. However, their analysis included very few genotype 2 patients: one included 23 genotype 2 patients and the other had 43 patients.

The distribution of HCV genotypes in Japan includes about 70% genotype 1b, with the remaining 30% genotypes 2a and 2b^[18]. The SVRs to treatment of even shorter duration have not yet been reported for Japanese patients. Data are needed to define whether or not the duration of treatment with IFN alpha in combination with ribavirin can be reduced from 24 wk without compromising antiviral efficacy in patients chronically infected with HCV of genotype 2. This investigation has assessed the efficacy of a 24-wk combination treatment of IFN alpha and ribavirin for Japanese patients with HCV genotype 2 infection and focussed on the issue of the relationship between the duration of treatment and the efficacy.

MATERIALS AND METHODS

Patients

A retrospective study was done on Japanese patients treated between December 2000 and March 2004 that included 173 patients, 20 years or older, who satisfied the following criteria: (1) chronically infected with HCV genotype 2a or 2b; and (2) a history of an increased alanine aminotransferase (ALT) level for over 6 months. Criteria for exclusion were: (1) clinical or biochemical evidence of hepatic decompensation; (2) hemoglobin level less than 115 g/L, white blood cell count less than 3×10^9 /L, and platelet count less than 50×10^9 /L; (3) concomitant liver disease other than hepatitis C (hepatitis B surface antigen or human immunodeficiency virus-positive); (4) alcohol or drug abuse; (5) suspected hepatocellular carcinoma; (6) severe psychiatric disease; and (7) treatment with antiviral or immunosuppressive agents prior to enrolment. Patients who fulfilled the above criteria were recruited at Kyushu University Hospital and 32 affiliated hospitals in the

northern Kyushu area of Japan.

Informed consent was obtained from all the patients before enrollment in this study. The study was approved by the institutional Ethics Committees of the hospitals involved and conducted in accordance with the ethical guidelines of the Declaration of Helsinki and the International Conference on Harmonization of guidelines for good clinical practice.

Study design

All patients were treated with 6-10 MU of IFN alpha-2b (Intron A; Schering-Plough, Osaka, Japan) subcutaneously daily for the first 2 wk, then thrice a week for 22 wk. Ribavirin (Rebetol; Schering-Plough) was administered orally for 24 wk at a daily dose of 600-800 mg based on the body weight (600 mg for patients weighing less than 60 kg and 800 mg for those weighing 60 kg or more). The above duration and dose were approved by the Japanese Minister of Health, Labour and Welfare. The 48-wk combination treatment and the ribavirin dosage of 1 000-1 200 mg recommended by the international guidelines were not permitted under the rules of the Japanese national health insurance system during the period of this study. The dose of ribavirin was reduced by 200 mg if the hemoglobin level fell to 100 g/L. Patients were considered to have ribavirin-induced anemia if the hemoglobin level decreased to less than 100 g/L. In such cases, a reduction in the dose of ribavirin was required. Both IFN alpha-2b and ribavirin were discontinued if the hemoglobin level, white blood cell count, or platelet count fell below 85 g/L, 1×10^9 /L, and 2.5×10^9 /L, respectively. The treatment was also discontinued if severe malaise developed, the continuation of treatment was judged not to be possible by the attending physician, or the patient desired to discontinue treatment.

Grouping by continuation or discontinuation of treatment

Patients were divided into the following four categories: Group A, patients who well tolerated the 24-wk combination treatment with IFN and ribavirin without a reduction in the dose of either drug; Group B, patients who received the full 24-wk combination treatment but who needed a reduction of the dose of IFN or ribavirin, or both; Group C, patients who discontinued the ribavirin treatment but continued the 24-wk IFN treatment; and Group D, patients who did not complete the 24 wk of treatment, because of adverse effects or who dropped out.

Determination of HCV RNA and HCV genotype and serotype

The serum HCV RNA level was examined with an Amplicor HCV monitor assay (version 2.0) (Roche, Tokyo, Japan), with a lower limit of quantitation of 500 IU (135 copies/mL) and an outer limit of quantitation of 850 000 IU/mL. Samples with HCV RNA over the limit of 850 000 IU/L were not diluted to determine the levels between 850 000-5 000 000 IU/mL. HCV RNA was also examined with the qualitative Amplicor HCV assay (Roche). HCV genotype was determined by type-specific primer from the core region of the HCV genome. The protocol for genotyping was carried out as described earlier^[11,12].

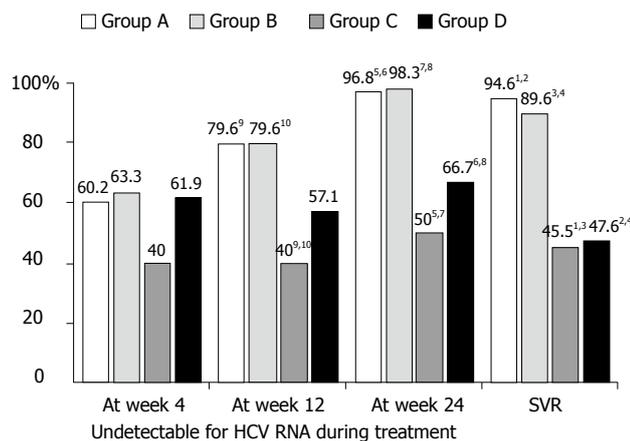


Figure 1 The sustained virological response (SVR) rate and undetectable hepatitis C virus (HCV) RNA rates during the treatment of 173 patients, classified by continuation and discontinuation of interferon and ribavirin combination treatment. Group A patients ($n=93$) who well tolerated the 24-week treatment with IFN and ribavirin in combination without any reduction in the dose of either drug; Group B patients ($n=48$) received the 24-week combination treatment, but needed a dose reduction of IFN or ribavirin, or both; Group C patients ($n=11$) discontinued the ribavirin treatment, but continued the full 24 weeks of IFN treatment; Group D patients ($n=21$) did not complete the 24 weeks of treatment because of adverse effects ($n=17$) or dropped out ($n=4$). ¹ $P=0.0001$; ² $P<0.0001$; ³ $P=0.0031$; ⁴ $P=0.0003$; ⁵ $P=0.0001$; ⁶ $P=0.0001$; ⁷ $P=0.0002$; ⁸ $P=0.0003$; ⁹ $P=0.0002$; ¹⁰ $P=0.0182$.

Histological examination

Liver biopsy was done for 117 patients infected with genotype 2 within the 6 months before the start of the treatment. For each specimen, a stage of fibrosis and a grade of activity were established according to the following criteria. Fibrosis was staged on a scale of 0-4: F0=no fibrosis, F1=portal fibrosis without septa, F2=few septa, F3=numerous septa without cirrhosis, F4=cirrhosis. The grading of activity, including the intensity of the necroinflammation, was scored as follows: A0=no histological activity, A1=mild activity, A2=moderate activity, A3=severe activity. Liver biopsy was not available from 56 patients who declined to have a biopsy.

Efficacy of treatment

The SVR was defined as undetectable HCV RNA by the qualitative Amplicor HCV assay (Roche) and a normal ALT level (under 40 IU/L) at 6 months after the end or stoppage of the treatment. Patients not achieving a SVR were considered as non-SVR. Patients who had undetectable HCV RNA within 4 wk of the start of treatment were considered to have had an early virological response (EVR).

Statistical analysis

The analysis of SVR was done on an intention-to-treatment basis, including dropouts, who were counted as non-sustained virological responders, and patients who stopped treatment. The χ^2 test or Fisher's exact test was used to examine the association between baseline characteristics and SVR. The Mann-Whitney U test was also used to compare responders and non-responders with regard to various characteristics, when appropriate. Independent factors associated with SVR were studied using forward

stepwise logistic regression analysis of the variables. Forward stepwise logistic regression analysis was done using a commercially available software package (BMDP Statistical Software Inc., Los Angeles, CA, USA) for the IBM 3090 system computer. A P -value of less than 0.05 was considered significant. All P -values were two tailed.

RESULTS

Patient characteristics, dose reduction and discontinuation of treatment regimen

The distribution of Groups A, B, C, and D patients was 93 (53.8%), 48 (27.7%), 11 (6.4%), and 21 (12.1%), respectively. Completing the 24-week ribavirin treatment were 141 patients in Groups A and B. Thirty-two patients of Groups C and D discontinued the ribavirin treatment.

The pretreatment characteristics of these four groups of patients are summarized in Table 1 and 2. The median age was significantly younger in Group A (51 years) than in Groups B (56 years) and C (59 years). Significantly more men were in Group A (71.0%) than in Group B (33.3%). The median creatinine clearance was significantly higher in Group A (110 mL/min) than Groups B (92 mL/min) and C (85 mL/min). The median hemoglobin level was significantly higher in Group A (150 g/L) than Groups B (136 g/L), C (134 g/L), and D (134 g/L). The median platelet count was significantly higher in Group A ($168 \times 10^9/L$) than in Group C ($127 \times 10^9/L$). No notable differences between the groups were found in body weight, ribavirin dose, HCV RNA level, genotype, or histology.

Virological response

SVR was achieved by 146 (84.4%) of 173 patients. The SVR did not differ between patients with genotypes 2a and 2b (83.1% vs 84.6%). The SVRs were 82.4% (14 of 17) (under 100 kIU/mL), 84.2% (16 of 19) (100-199 kIU/mL), 85.7% (24 of 28) (200-299 kIU/mL), 83.3% (15 of 18) (300-399 kIU/mL), 100% (12 of 12) (400-499 kIU/mL), 76.9% (10 of 13) (500-599 kIU/mL), 77.8% (7 of 9) (600-699 kIU/mL), 90.9% (10 of 11) (700-799 kIU/mL), and 82.6% (38 of 46) (800 and over kIU/mL). The SVRs were 76.9-100%. The SVRs of the HCV genotype 2 patients with any level of viremia level did not significantly differ.

Figure 1 shows the SVR and undetectable HCV viremia rate during the treatment of 173 patients, classified by continuation and discontinuation of combination treatment. The SVRs were significantly higher in Groups A (94.6%) and B (89.6%) than in Groups C (45.5%) and D (47.6%). A significant difference of SVR was found between patients with and without discontinuation of ribavirin (46.9%, 15 of 32 of Groups C and D patients vs 92.9%, 131 of 141 of Groups A and B patients, $P<0.0001$). During the treatment period, except for at week 4, the rates of undetectable HCV RNA were also significantly higher in Groups A and B than in Groups C and D.

Figure 2 shows the relationship between SVR and the ribavirin treatment period in all the patients. A significant difference was found between patients with less than 16 wk of treatment period and patients with longer periods

Table 1 Baseline characteristics

| Characteristic | Complete Ribavirin treatment (n = 141) | | Discontinued Ribavirin treatment (n = 32) | | All patients (n = 173) |
|--|---|-----------------------------|--|---------------------|---------------------------|
| | Group A (n = 93) | Group B (n = 48) | Group C (n = 11) | Group D (n = 21) | |
| Median age (yr) (range) | 51 ^{1,2} (20-73) | 56 ¹ (25-70) | 59 ² (53-73) | 50 (29-73) | 53 (20-73) |
| Male (%) | 66 (71.0) ³ | 16 (33.3) ³ | 5(50.0) | 13 (61.9) | 100 (57.8) |
| Body weight | | | | | |
| 60 kg or more (%) | 60 (64.5) | 23 (47.9) | 6 (54.5) | 12 (57.1) | 101 (58.3) |
| Ribavirin dose by weight | | | | | |
| 12 mg/kg or more (%) | 24 (25.8) | 20 (41.7) | 4 (36.4) | 9 (42.8) | 57 (32.9) |
| Creatinine clearance (mL/min) (range) | 110 ^{4,5} (53-261) | 92 ⁴ (46-167) | 85 ⁵ (60-111) | 101 (41-203) | 102 (41-261) |
| HCV RNA level | | | | | |
| 500 kIU/mL or more (%) | 44 (47.3) | 22 (45.8) | 4 (36.4) | 9 (42.8) | 79 (45.7) |
| Genotype 2a (%) | 67 (72.0) | 28 (58.3) | 6 (54.5) | 13 (61.9) | 114 (65.9) |

¹P=0.0401; ²P=0.0044; ³P<0.0001; ⁴P=0.0002; ⁵P=0.0248

Table 2 Baseline characteristics (continued)

| Characteristic | Complete Ribavirin treatment (n = 141) | | Discontinued Ribavirin treatment (n = 32) | | All patients (n = 173) |
|--|---|-------------------------------|--|--------------------------------|---------------------------|
| | Group A (n = 93) | Group B (n = 48) | Group C (n = 11) | Group D (n = 21) | |
| Histology | | | | | |
| Stage of fibrosis | | | | | |
| F0 - F1 (%) | 27 (43.5) | 17 (50.0) | 4 (50.0) | 9 (42.9) | 56 (47.9) |
| F2 - F3 (%) | 35 (56.5) | 15 (44.1) | 4 (50.0) | 6 (28.6) | 59 (50.4) |
| F4 (%) | 0 - | 2 (5.9) | 0 - | 0 - | 2 (1.7) |
| Not determined | 31 | 14 | 3 | 6 | 54 |
| Grade of activity | | | | | |
| A0 - A1 (%) | 27 (43.5) | 17 (50.0) | 4 (50.0) | 9 (42.9) | 43 (47.9) |
| A2 (%) | 35 (56.5) | 15 (44.1) | 4 (50.0) | 6 (28.6) | 58 (50.4) |
| A3 (%) | 0 - | 2 (5.9) | 0 - | 0 - | 16 (1.7) |
| Not determined | 31 | 14 | 3 | 6 | 54 |
| Median hemoglobin (g/L) (range) | 150 ^{6,7,8} (117-171) | 136 ⁶ (116-163) | 134 ⁷ (121-152) | 134 ⁸ (12.1-153) | 144 (116-171) |
| Median platelet count (X 10 ⁹ /L) (range) | 168 ⁹ (79-385) | 167 (58-363) | 127 ⁹ (55-181) | 157 (57-240) | 162 (55-385) |

⁶P=0.0003; ⁷P=0.0063; ⁸P=0.0225; ⁹P=0.0120

(34.8%, 8 of 23 *vs* 92.0%, 138 of 150, $P < 0.0001$), showing that 16 wk of ribavirin treatment significantly contributed to a SVR. Of the 173 studied patients, 104 (60.1%) had an EVR, defined as undetectable HCV RNA within 4 wk of the start of treatment. The SVR was 94 (90.4%) of these 104 patients with EVR, which was significantly higher than the non-EVR patients (52 of 69, 75.4%) ($P = 0.0142$). No significant differences were found between patients with and without undetectable HCV RNA at 8 or 12 wk of the start of treatment. Moreover, we analyzed the relationship between SVR and the length of ribavirin treatment in the 104 patients with EVR. A significant difference was found between patients with less than 16 wk of ribavirin treatment and those with a longer treatment period (46.2%, 6 of 13 *vs* 96.7%, 88 of 91, $P < 0.0001$). These findings

showed that 16 wk of ribavirin treatment significantly contributed to a SVR, even in patients with EVR.

Factors contributing to SVR

To assess the independent role of the IFN and ribavirin combination treatment on SVR, an adjustment by forward stepwise logistic regression analysis for all other independent risk factors identified was done. The continuation of ribavirin treatment ($P < 0.0001$) was significantly associated with SVR in analysis of all the patients. A higher SVR (odds ratio = 13.15) was found for patients who continued to receive ribavirin treatment than for those who discontinued it. Other factors such as sex, age, HCV genotype, pretreatment-HCV RNA level, histological findings, pretreatment platelet count and creatinine clearance, history

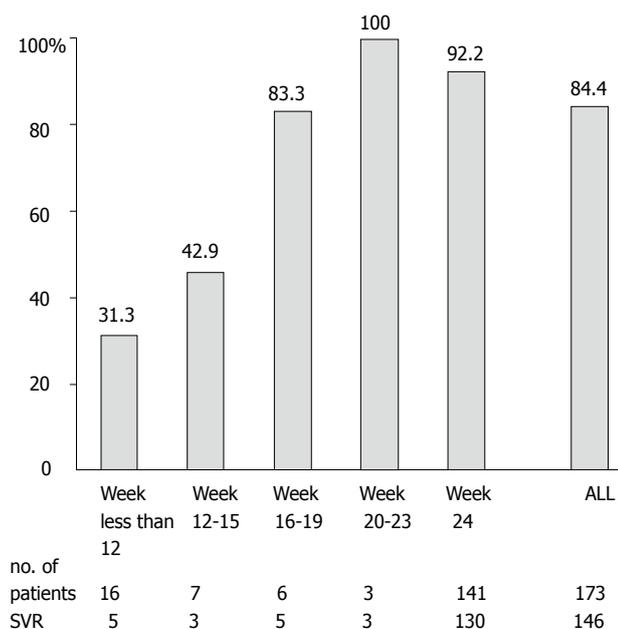


Figure 2 Relationship between the sustained virological response rates and the length of ribavirin treatment period of the 173 studied patients.

of prior IFN, and dose reduction of IFN or ribavirin were not significantly, independently associated with a SVR.

DISCUSSION

The large number of Japanese HCV genotype 2 patients enrolled in this study was sufficient to provide for meaningful statistical analysis, even though it was retrospective. This study demonstrated that a 24-wk IFN and ribavirin combination treatment was highly effective and resulted in a remarkably high SVR (84.4%) in genotype 2 patients, as expected. Importantly, we also showed that dose reductions of ribavirin were not associated with a poor outcome in these patients, only ribavirin discontinuation, and that the addition of ribavirin for up to 16 wk contributed to the high SVR.

In December 2004, pegylated IFN plus ribavirin combination treatment received the official approval in Japan. The combination treatment was not yet approved for clinical use for patients with chronic HCV viremia by the Japanese Ministry of Health, Labour and Welfare at the time of the present study. So far, our most effective and available treatment is the 6-month IFN-alpha plus ribavirin combination.

Remarkably high SVRs were observed for our patients with genotype 2 who took the IFN and ribavirin combination treatment. IFN monotherapy does not result in a satisfactory outcome for patients with chronic hepatitis C, particularly those with genotype 1, which is known to be IFN-resistant, whereas genotype 2 is IFN-sensitive^[11-13]. The addition of ribavirin, a synthetic purine nucleoside analog, to IFN enhances the virological response^[8,9,13-17]. Our research group, KULDS, also analyzed the data of patients with genotype 1 who were treated with this 24-wk combination treatment: SVR was achieved by 21% of 528 patients with genotype 1 by intention-to-treat analysis

(data not published). Differences between genotype 1 and 2 patients still existed following the ribavirin combination treatment. Moreover, a striking finding in our study was that there were no differences among the patients with genotype 2 of any HCV RNA level (76.9-100%). The precise mechanism is unclear, although it possibly originates in different nucleotide sequence of their genome. Further study is needed to clarify the reasons for the differences in antiviral effect, by the use of novel and new tools for the quantification of the HCV replication system^[19,20].

How long the ribavirin needs to be administered to achieve the best efficacy with IFN alpha-treated patients of genotype 2 is unclear. In the present study, SVR after 16 or more weeks of treatment ranged from 83.3% to 100% and was not dependent on the dose reduction of ribavirin treatment but on the discontinuation of IFN or ribavirin treatment. A pilot study from Norway showed that patients with genotype 2 and an EVR obtained a high SVR after 14 weeks of pegylated IFN and ribavirin combination treatment^[21]. The Zeuzem group also demonstrated a very high SVR in a 24-week pegylated IFN and ribavirin treatment for genotype 2 patients, and 16-week treatment duration was observed to be a significant independent predictor^[17]. In view of the adverse effects, high cost of ribavirin, and the above mentioned findings along with our results, a 16-week ribavirin addition to IFN treatment would seem to produce a high rate of SVR for patients with genotype 2, especially for those with EVR, defined as undetectable HCV RNA within 4 wk of the start of the treatment.

The Davis group attempted to confirm that an EVR in patients with chronic hepatitis C undergoing initial treatment with a combination therapy of pegylated IFN alpha and ribavirin was predictive of SVR^[22]. Retrospective analysis of data from other trials^[23] has also suggested that patients who do not attain EVR have a nominal chance of SVR with additional weeks of treatment. While the primary goal, or "holy grail", of treatment of chronic hepatitis C is SVR, it must be acknowledged there are other secondary goals that compel physicians to continue treatment without EVR. In fact, patients who do not achieve EVR or SVR may have histological benefit^[24], leading to a decreased risk of hepatocellular carcinoma^[19]. Thus, it remains to be determined whether or not early discontinuation of treatment would reduce economic costs if a long-term perspective is taken.

Several adverse reactions are associated with ribavirin. One of the most significant reactions is hemolytic problems, especially anemia^[15]. Most of our patients who had to have a dose reduction or who discontinued ribavirin were observed to have anemia. It is important to reduce the dose of ribavirin at as early a stage as possible to allow the safe continuation of the combination treatment. The Nomura group pointed out that careful administration is necessary in patients over 60 years, in female patients, and in patients receiving a ribavirin dose by body weight of 12 mg/kg or more^[21]. Our forward stepwise logistic regression analysis showed that the continuation of ribavirin treatment was significantly associated with SVR. This combination treatment, which could depend on hemolytic adverse reaction, has a high efficacy, if physicians are able to continue the ribavirin treatment for as short a period as

16 wk, even when taking into account of the dose reductions necessary for patients with a dangerous decrease of hemoglobin caused by ribavirin, as often seen in genotype 2 patients with a low hemoglobin level at pretreatment.

In conclusion, the 24-week IFN and ribavirin combination treatment was highly effective and resulted in a remarkably high SVR in Japanese HCV patients with genotype 2 from the retrospective study of ours. The most significant predictor was continuation of the ribavirin treatment for up to 16 wk. These findings are not pertinent to the other different genotypes.

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Omeprazole maintenance therapy prevents recurrent ulcer bleeding after surgery for duodenal ulcer

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Abstract

AIM: To evaluate the omeprazole maintenance therapy in patients with recurrent ulcer bleeding after surgery for duodenal ulcer.

METHODS: We studied 15 consecutive patients with recurrent ulcer bleeding after surgery for duodenal ulcer. Omeprazole (20 mg/d) maintenance therapy was given after ulcer healing. In addition to clinical follow-up, ambulatory 24-h gastric pH assay was performed before and during omeprazole therapy in those patients and controls with previous duodenal ulcer surgery but no ulcer recurrence.

RESULTS: All the 15 ulcers were healed after being treated with omeprazole (40 mg/d) for 2 mo. Eleven patients with two (1-9) episodes of recurrent ulcer bleeding completed the follow-up (43, 12-72 mo). None of them had a bleeding episode while on omeprazole. One patient discontinued the therapy and had recurrent bleeding. The median 24-h fraction time of gastric pH <4 in patients was 80, 46-95%, and was reduced to 32, 13-70% by omeprazole ($P=0.002$).

CONCLUSION: Long-term maintenance therapy with omeprazole (20 mg/day) is effective in preventing recurrent ulcer bleeding.

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Key words: Duodenal ulcer; Gastrectomy; Vagotomy; Omeprazole

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INTRODUCTION

H pylori eradication therapy and the use of potent proton pump inhibitors (PPIs) have dramatically reduced the need for surgical therapy of peptic ulcer disease. Still, about 10% of duodenal ulcer patients undergo emergency surgical therapy for acute ulcer bleeding^[1]. However, recurrent ulcer is not uncommon as it occurs in 10-15% of patients after vagotomy and drainage and in 2-5% of patients after gastric resection^[2]. This may be complicated by life threatening acute recurrent ulcer bleeding in certain patients, requiring hospitalization.

Several studies have investigated the rate of ulcer recurrence after duodenal ulcer surgery^[2,3] and the completeness of vagotomy^[4,5], but only a few studies have evaluated the anastomotic ulcer healing rates after being treated with H₂ receptor antagonists (H₂RA)^[6,7] or PPI^[8] therapy. Studies have shown that *H pylori* infection of the gastric mucosa is not related to ulcer recurrence after gastric surgery^[4,9,10]. Furthermore, it has been shown that 28% of anastomotic ulcers recur within 6 wk after discontinuing cimetidine therapy^[7], and 33% relapse within a year while on cimetidine maintenance therapy^[6].

These patients are often treated with a second operation^[1]. However, to the best of our knowledge, there are no studies investigating the long-term outcome of patients with recurrent post-surgical ulcer and whether maintenance acid suppression therapy with PPIs may prevent recurrent ulceration and/or re-bleeding. Therefore, the present prospective open label study was conducted to investigate gastric pH profile and the effect of omeprazole maintenance therapy in patients presented with recurrent ulcer bleeding after duodenal ulcer surgical therapy.

MATERIALS AND METHODS

Over a 7-year period, this prospective open label study included 15 consecutive male patients admitted to our department due to recurrent acute ulcer bleeding. All patients underwent gastric surgery for duodenal ulcer disease at least 2 years ago.

Clinical study

In each case, emergency endoscopy was performed to confirm recurrent ulcer bleeding. The finding of an ulcer was considered as the bleeding cause if active bleeding or stigmata of recent hemorrhage were noted in the absence of other lesions. The recurrent ulcers were peristomal or duodenal in location. At the same time, detailed history was obtained about the indication and time of past gastric operation and the number of hospital admissions with hematemesis or melena after gastric surgery. History specifically included questions about the use of H₂RA, PPIs or non-steroidal anti-inflammatory drugs (NSAIDs)^[11], smoking and alcohol abuse. In all the patients fasting serum gastrin and salicylate concentrations were determined to exclude Zollinger–Ellison syndrome and recent consumption of non-steroidal anti-inflammatory drugs. Patients who were on non-steroidal anti-inflammatory drugs were excluded. During endoscopy, multiple gastric mucosal biopsies were obtained to investigate *H pylori* infection.

All patients were initially treated with intravenous omeprazole (20 mg every 12 h) and then orally after discharge from the hospital. *H pylori* eradication therapy was not used to prevent ulcer recurrence^[10,12], but *H pylori* was eradicated in two patients because of severe *H pylori* gastritis. Follow-up endoscopy was scheduled at 2 mo, while on oral omeprazole (40 mg/d) to confirm ulcer healing. Thereafter, the patients were instructed to receive oral omeprazole (20 mg/d) maintenance therapy, to avoid the use of any non-steroidal anti-inflammatory drugs and to have follow-up every 6 mo as outpatients.

Twenty-four-hour gastric pH studies

Twenty-four-hour gastric pH studies were performed in the following groups on omeprazole therapy (20 mg/d) but not on antisecretory therapy: patients with first or second degree reflux esophagitis (Los Angeles classification) (“normal” controls); patients with duodenal ulcer; controls who underwent vagotomy and pyloroplasty or gastrojejunostomy for duodenal ulcer but had no ulcer recurrence; controls who had Billroth II partial gastrectomy and patients who had recurrent anastomotic ulcer bleeding after gastric surgery for duodenal ulcer. In the latter group of patients (test group), 24-h gastric pH studies were performed while on omeprazole (40 mg/d, 20 mg/d). Omeprazole was then discontinued and the patients were treated with ranitidine 150 mg twice daily for 2 d, followed by a 2-d washout period before the pH study in patients not on antisecretory therapy. The duodenal ulcer group included patients admitted to our department for acute ulcer bleeding and volunteered to have 24-h pH studies. The vagotomy and gastrectomy control groups included patients who attended the outpatient clinic for various epigastric symptoms and volunteered to participate in the study after having a negative gastroscopy.

Gastric ambulatory pH monitoring was performed using a monocrySTALLINE antimony pH catheter. The electrode was passed transnasally into the stomach, 10-15 cm below the detectable esophagogastric junction by endoscopy. The catheter was connected to a portable pH-recording device

(Digitrapper Mk III, Synectics Medical AB, Stockholm, Sweden). Recorded data were uploaded into the “EsopHogram Analysis Software” for analysis and review. During the 24-h pH studies, patients were encouraged to keep up their usual activities and diet in order to maximize the diagnostic yield of the test. The 24-h fraction (%) time when the gastric pH was below 4 was calculated.

All patients and volunteers gave oral consent after being informed of the purpose of the study by the investigator. The study protocol was approved by the Ethics Committee on Human Studies, Department of Internal Medicine, Medical School of the Athens University. The trial was conducted according to the declarations of Helsinki.

Statistical analysis

We used non-parametric statistics [“Statgraphics-Plus” version 4 for Windows (Manugistics Inc., Rockville, USA)], because of the small number of observations included in each group. Results were presented as median with ranges. We used the non-parametric Mann-Whitney (Wilcoxon) two-sided *U*-test and the Kruskal-Wallis *t*-test for two and multiple-sample comparison analysis, respectively^[13]. *P* < 0.05 was considered statistically significant.

RESULTS

Clinical data and follow-up

Over a 7-year period, 15 consecutive male patients with recurrent ulcer bleeding after surgery for duodenal ulcer were admitted to our department because of acute bleeding episode. Serum gastrin was normal and salicylates were not detected in the serum in any of the 15 patients during their hospitalization. In addition, all patients denied the use of non-aspirin, non-steroidal anti-inflammatory drugs after thorough interrogation.

Ulcer in all the 15 patients was healed after treatment with omeprazole (40 mg/d) for 2 mo. However, four of them did not consent to gastric pH studies and were lost to clinical follow-up within the first year from entering the study. Therefore, data were presented for 11 patients (Table 1).

Two out of the eleven patients had a history of two gastric operations because of recurrent ulcer bleeding. The time of recurrent ulcer bleeding after gastric surgery was 6 years, averaged 2-18 years. The number of hospital admissions because of post surgical ulcer bleeding was 2, averaged 1-9. At study entry, 3/11 patients were smokers and 5/11 were *H pylori*-positive. *H pylori* was successfully eradicated in two of these five patients upon admission to the study. One of the *H pylori*-negative patients had successful triple eradication therapy at another hospital a year ago.

No recurrent ulcer or re-bleeding occurred during the maintenance therapy (omeprazole 20 mg/d) over the follow-up period (43 mo, averaged 12-72 mo). One patient discontinued treatment and had recurrent bleeding 8 mo after stopping omeprazole. Currently, he was symptom-free on omeprazole maintenance therapy. There were no significant adverse events related to treatment.

Table 1 Background information of patients who had recurrent ulcer bleeding after surgery for peptic ulcer disease

| Case | Age (yr) | Smoking status | <i>H. pylori</i> status | Type of operation | Years since operation | Bleeding episodes after surgery | Follow-up (mo) |
|------|----------|----------------|-------------------------|--------------------|-----------------------|---------------------------------|----------------|
| 1 | 62 | Never | Positive ¹ | TV+D ² | 5, 2 ³ | 3 | 72 |
| 2 | 60 | Ex-smoker | Positive | TV+D | 6 | 8 | 62 |
| 3 | 61 | Smoker | Positive ¹ | BII | 2 | 1 | 61 |
| 4 | 66 | Never | Negative | BII+V ² | 4, 3 ³ | 2 | 56 |
| 5 | 49 | Smoker | Positive | TV+D | 7 | 1 | 52 |
| 6 | 70 | Ex-smoker | Negative | BII | 18 | 2 | 43 |
| 7 | 64 | Never | Negative | BII | 3 | 2 | 35 |
| 8 | 59 | Ex-smoker | Negative | BII | 16 | 4 | 32 |
| 9 | 66 | Smoker | Negative | TV+D | 7 | 5 | 28 |
| 10 | 52 | Ex-smoker | Positive | TV+D | 5 | 2 | 18 |
| 11 | 48 | Ex-smoker | Negative | TV+P | 13 | 9 | 12 |

¹Successful *H. pylori* eradication therapy upon entering the study. ²Final operation. ³Years elapsed since the first and second gastric operation, upon entering the study. TV+D: truncal vagotomy and gastrojejunostomy. TV+P: truncal vagotomy and pyloroplasty. BII: Billroth II partial gastrectomy.

Twenty-four-hour gastric pH studies

Results of the 24-h intragastric pH monitoring studies are shown in Table 2. The "normal" control group included 10 patients with first or second degree reflux esophagitis, who were matched for age, sex and *H. pylori* status with the recurrent post-surgical ulcer patients. They were selected from a pool of patients with reflux esophagitis on 24-h esophageal-gastric pH monitoring. The duodenal ulcer control group included seven patients hospitalized because of duodenal ulcer bleeding. The vagotomy control group included seven patients who had truncal vagotomy and drainage for duodenal ulcer. The gastrectomy control group included seven patients who had partial gastrectomy (Billroth II) for duodenal ulcer. Volunteers of the vagotomy and gastrectomy control groups studied at least 2 years postoperatively had no postoperative ulcer recurrence. The test group included ten of the eleven patients who had truncal vagotomy ($n=5$) or Billroth II gastrectomy ($n=5$) and recurrent anastomotic ulcer bleeding. Intolerance to nasogastric pH probe was not studied in another patient who had vagotomy and drainage. There were three failures out of 30 24-h gastric pH recordings in the test group, as shown by missing values in Figure 1. One of the vagotomy controls refused to have the 24-h pH study on omeprazole.

Treatment-free (baseline) 24-hour gastric pH studies

The 24-h gastric pH measurements of the groups studied during treatment-free period are shown in Table 2. There was a statistically significant difference in the 24-h fraction time of gastric pH <4 among the six groups ($T=29.10$, $P<0.001$), with the lowest values recorded in the Billroth II gastrectomy control group. Patients who had vagotomy and recurrent ulcer bleeding had a significantly different (higher) 24-h fraction time of gastric pH <4 as compared to vagotomy controls ($U=32$, $P=0.023$). This difference was also significant when bleeding patients after gastrectomy were compared to those of the gastrectomy

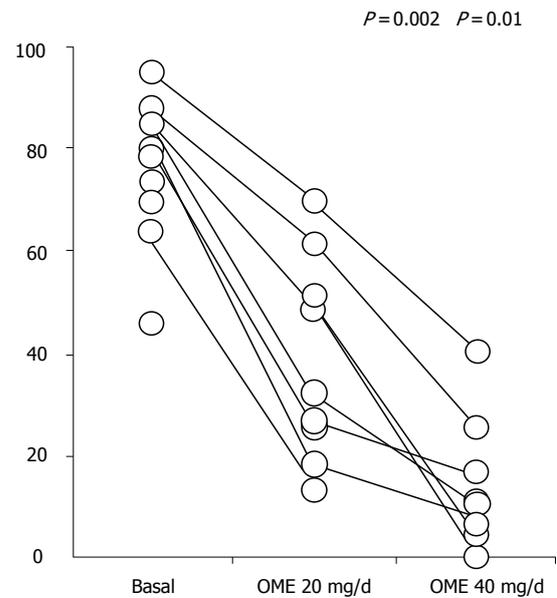


Figure 1 Individual data points of the 24-h intra gastric pH monitoring studies while on no treatment (baseline), and during therapy with 20 or 40 mg/d omeprazole (OME) in patients with recurrent post-surgical ulcer bleeding.

control group ($U=35$, $P=0.006$).

Twenty-four hour gastric pH studies (omeprazole 20 mg/day)

Gastric pH studies were performed in the control and patient (test) groups on omeprazole (20 mg/d). Billroth II gastrectomy control group did not undergo this study because of the low fraction time of gastric pH <4 recorded during treatment-free gastric pH monitoring. Omeprazole therapy (20 mg/d) significantly reduced the 24-h fraction time of gastric pH <4 in both control and patient groups (Table 2).

Twenty-four hour gastric pH studies in patient group

Eight out of the ten patients underwent all (baseline 20 mg/day and 40 mg/day omeprazole) 24-h gastric pH studies. One additional patient underwent only baseline, and another patient both baseline and omeprazole (20 mg/day) 24-h gastric pH studies. Individual data are shown in Figure 1. The 24-h fraction time of gastric pH <4 (80, 46-95%) was significantly reduced by omeprazole (20 mg/d) to 32, 13-70% ($U=6$, $P=0.002$) and by omeprazole (40 mg/d) to 11, 1-40% (omeprazole 20 mg/day *vs* 40 mg/day, $U=9$, $P=0.01$).

DISCUSSION

The data of the present study showed that recurrent ulcers after gastric surgery for duodenal ulcer can heal after 2 months of 40 mg/day omeprazole therapy. Even more important, none of our patients had a re-bleeding episode while on omeprazole maintenance therapy over a 3.5-year follow-up period.

A large review is available on the complications associated with ulcer recurrence following gastric surgery including 130 studies published on this topic over a

Table 2 Demographic data of patients and control groups and results (median, range) of the 24-h fraction (%) time of gastric pH < 4 before (basal) and while on 20 mg/d omeprazole.

| Groups studied | Number of patients | Age (yr) | <i>H. pylori</i> (+) | Treatment-free (baseline) | Omeprazole 20 mg/d | <i>P</i> value |
|--------------------------------|--------------------|-----------|----------------------|---------------------------|--------------------|----------------|
| Normal controls | 10 | 60, 47–73 | 2/9 | 87.7, 71.3–98.5 | 34.6, 4.3–72.7 | <0.001 |
| Duodenal ulcer controls | 7 | 61, 38–71 | 7/7 | 92.3, 86.6–98.8 | 45.2, 25.8–72.0 | 0.002 |
| Vagotomy controls ² | 7 | 54, 41–72 | 2/6 | 72.8, 34.0–87.3 | 17.8, 6.5–35.1 | 0.005 |
| Gastrectomy controls | 7 | 66, 46–72 | 1/7 | 6.2, 4.6–39.6 | ND ¹ | |
| Vagotomy patients | 5 | 57, 49–72 | 3/5 | 84.9, 79.1–95.2 | 49.4, 18.3–69.9 | 0.012 |
| Gastrectomy patients | 5 | 68, 61–72 | 0/5 | 69.8, 45.8–85.2 | 28.8, 13.3–51.3 | 0.037 |

¹ND, not done. ²Truncal vagotomy and gastrojejunostomy (*n*=5), truncal vagotomy and pyloroplasty (*n*=2).

30-year period^[14]. The authors estimated that vagotomy plus drainage is associated with a 9% recurrence rate and a risk of hemorrhage of 1.7%. Partial gastrectomy has a lower recurrence rate (<1%) but a similar risk of hemorrhage (1.3%). Such patients with recurrent ulcer bleeding are often submitted to a second gastric operation to cure recurrent ulcer. Similarly, most of our patients had a history of two or more hospital admissions for recurrent ulcer bleeding after surgery and two of them had a second gastric operation that failed to cure recurrent ulcer.

Cimetidine has been used in a few clinical trials including a small number of patients with recurrent ulcer bleeding following surgery for peptic ulcer disease^[6,7,15-17]. Cimetidine heals about 85% of ulcers within 8 weeks^[17], but about 30% of ulcers relapse within 6 months after discontinuing therapy^[7,17]. Furthermore, 33% of ulcers recur 1 year after surgery while on cimetidine maintenance therapy^[6], probably because of tolerance to H₂RA, which is more significant after 4 wk of therapy^[18]. Finally, there is only one clinical trial using omeprazole therapy for 12 patients with recurrent ulcer after vagotomy or gastrectomy and all ulcers are healed after 8 weeks of omeprazole therapy^[8], suggesting that maintenance therapy with PPIs is the only alternative for surgical intervention. Since as many as one-third of patients with anastomotic ulcer have recurrent ulcer within a year after discontinuing acid reduction therapy^[6,15], an open trial of PPI maintenance treatment should be considered ethically justifiable.

The role of *H. pylori* in recurrent peptic ulcer disease after gastric surgery has been investigated in several clinical studies^[9,10,12,19], showing that *H. pylori* does not play an important role in post surgical ulcer recurrence. Less than half of our patients with recurrent ulcer bleeding were *H. pylori*-positive upon admission to the study. Furthermore, one of our patients who had successful *H. pylori* eradication therapy during a previous hospitalization was admitted to our department for a new episode of anastomotic ulcer bleeding. Another patient had two gastric operations for recurrent ulcer bleeding. Upon study entry, he had successful *H. pylori* eradication therapy, followed by omeprazole (20 mg/d) maintenance therapy. He was healthy for 2 years and then lost his follow-up. He was readmitted to another hospital for recurrent ulcer bleeding 8 mo after discontinuing PPI maintenance therapy, though he was *H. pylori*-negative.

With regard to the underlying mechanism of ulcer recurrence, our study showed that patients with recurrent ulcer bleeding after truncal vagotomy or partial gastrectomy had a higher treatment-free 24-h intragastric acidity than controls with no ulcer recurrence after the same operation, suggesting that vagotomy may be incomplete due to a retained antrum^[20,21]. Our data showed that ulcer healing therapy with omeprazole (40 mg/d) could strongly inhibit gastric acid secretion. The intragastric acidity could also be significantly reduced with oral omeprazole maintenance treatment (20 mg/d).

Other factors that may be implicated in ulcer recurrence after surgical treatment for duodenal ulcer include a decreased resistance to acid in the jejunal mucosa, the site of pyloroplasty and anastomosis, possibly due to local ischemia and scarring. Hirschowitz and Lanos^[11] also showed that aspirin abusers develop intractable recurrent ulceration following gastric surgery for peptic ulcer disease. However, our patients refused to use non-steroidal anti-inflammatory drugs and their serum salicylates were negative. Zollinger-Ellison syndrome was also excluded by normal fasting serum gastrin.

In conclusion, omeprazole (20 mg/d) maintenance therapy should be the treatment of choice for patients with recurrent ulcer after gastric surgery for duodenal ulcer disease.

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RAPID COMMUNICATION

Involvement of mitochondrial $\text{Na}^+-\text{Ca}^{2+}$ exchange in intestinal pacemaking activity

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Abstract

AIM: Interstitial cells of Cajal (ICCs) are the pacemaker cells that generate slow waves in the gastrointestinal (GI) tract. We have aimed to investigate the involvement of mitochondrial $\text{Na}^+-\text{Ca}^{2+}$ exchange in intestinal pacemaking activity in cultured interstitial cells of Cajal.

METHODS: Enzymatic digestions were used to dissociate ICCs from the small intestine of a mouse. The whole-cell patch-clamp configuration was used to record membrane currents (voltage clamp) and potentials (current clamp) from cultured ICCs.

RESULTS: Clonazepam and CGP37157 inhibited the pacemaking activity of ICCs in a dose-dependent manner. Clonazepam from 20 to 60 $\mu\text{mol/L}$ and CGP37157 from 10 to 30 $\mu\text{mol/L}$ effectively inhibited Ca^{2+} efflux from mitochondria in pacemaking activity of ICCs. The IC_{50} s of clonazepam and CGP37157 were 37.1 and 18.2 $\mu\text{mol/L}$, respectively. The addition of 20 $\mu\text{mol/L}$ NiCl_2 to the internal solution caused a "wax and wane" phenomenon of pacemaking activity of ICCs.

CONCLUSION: These results suggest that mitochondrial $\text{Na}^+-\text{Ca}^{2+}$ exchange has an important role in intestinal pacemaking activity.

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Key words: Mitochondrial $\text{Na}^+-\text{Ca}^{2+}$ exchange; Interstitial cells of Cajal

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INTRODUCTION

The interstitial cells of Cajal (ICCs) produce spontaneous rhythmic inward currents that are critical for the generation of slow waves in intestinal smooth muscle^[1-3]. Pacemaker currents in ICCs result from the activation of a voltage-independent, non-selective cation conductance^[4,5]. Pacemaking activity in ICCs is dependent upon metabolic activity^[6] and Ca^{2+} release from intracellular stores^[7]. Recent findings suggested that the pacemaker conductance in ICC is regulated by intracellular Ca^{2+} modulation^[8]. The close association between IP_3 receptor-dependent Ca^{2+} stores, mitochondria, and ion channels in the plasma membrane creates a basic cellular structure^[9,10]. Release of Ca^{2+} from IP_3 receptors does not directly initiate pacemaker currents in ICC, but rather, initiates Ca^{2+} uptake into the mitochondria. It was found that mitochondria in ICCs experience Ca^{2+} oscillations at the same frequency as pacemaker currents and that a rise in mitochondrial Ca^{2+} slightly precedes the activation of pacemaker currents^[8]. This implies that pacemaker channels in the plasma membrane are activated by the falling phase of localized Ca^{2+} transients.

In isolated mitochondria, Ca^{2+} influx occurs via a Ca^{2+} uniporter driven by the membrane potential^[11]. Ca^{2+} efflux occurs via $\text{Na}^+-\text{Ca}^{2+}$ exchange and can be inhibited by diltiazem, clonazepam, CGP37157, and by high external Ca^{2+} ^[11-14]. However, the effect of inhibiting mitochondrial efflux, by using inhibitors of the $\text{Na}^+-\text{Ca}^{2+}$ exchange, on pacemaking activity of ICCs has not yet been investigated. Therefore, we undertook to investigate the involvement of mitochondrial $\text{Na}^+-\text{Ca}^{2+}$ exchange in pacemaking activity of ICCs.

MATERIALS AND METHODS

Preparation of cells and cell cultures

Balb/c mice (8-13 days old) of either sex were anesthetized with ether and killed by cervical dislocation. The small intestines from 1 cm below the pyloric ring to the cecum were removed and opened along the mesenteric border. Luminal contents were removed by washing with Krebs-Ringer bicarbonate solution. The tissues were pinned to

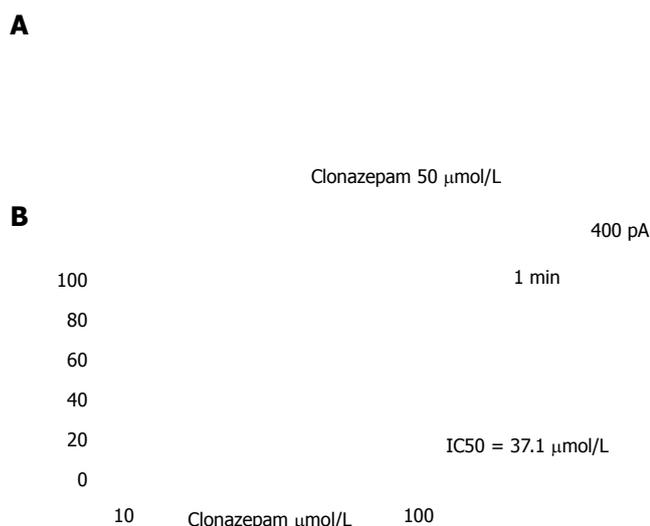


Figure 1 The effect of clonazepam on the pacemaking activity of ICCs. Clonazepam was applied to examine its effect on the pacemaking activity of ICCs. A: Under a voltage clamp at a holding potential of -60 mV, 50 $\mu\text{mol/L}$ clonazepam inhibited the pacemaking currents of ICCs ($n=4$). B: Clonazepam from 20 to 60 $\mu\text{mol/L}$ effectively inhibited Ca^{2+} efflux from mitochondria in the pacemaking activity of ICCs. The IC_{50} of clonazepam was 37.1 $\mu\text{mol/L}$.

the base of a Sylgard dish and the mucosa removed by sharp dissection. Small tissue strips of the intestine muscle (consisting of both circular and longitudinal muscles) were equilibrated in Ca^{2+} -free Hanks solution (containing in mmol/L: KCl 5.36, NaCl 125, NaOH 0.34, Na_2HCO_3 0.44, glucose 10, sucrose 2.9, and HEPES 11) for 30 min. Then, the cells were dispersed using an enzyme solution containing collagenase (Worthington Biochemical Co., Lakewood, NJ, USA) 1.3 mg/mL, bovine serum albumin (Sigma Chemical Co., St. Louis, MO, USA) 2 mg/mL, trypsin inhibitor (Sigma) 2 mg/mL and ATP 0.27 mg/mL. Cells were plated onto sterile glass coverslips coated with murine collagen (2.5 $\mu\text{g/mL}$, Falcon/BD, Franklin Lakes, NJ, USA) in a 35-mm culture dish and then cultured at 37°C in a 95% O_2 , 50 mL/L CO_2 incubator in a smooth muscle growth medium (Clonetics Corp., San Diego, CA, USA) supplemented with 2% antibiotics/antimycotics (Gibco, Grand Island, NY, USA) and murine stem cell factor (SCF, 5 ng/mL, Sigma). ICCs were identified immunologically with anti-c-kit antibody (phycoerythrin-conjugated rat anti-mouse c-kit monoclonal antibody; eBioscience, San Diego, CA, USA) at a dilution of 1:50 for 20 min^[15]. ICCs were morphologically distinct from other cell types in the culture and thus it was possible to identify the cells by phase contrast microscopy once they had been verified with anti-c-kit antibody.

Patch-clamp experiments

The whole-cell patch-clamp configuration was used to record membrane currents (voltage clamp) and potentials (current clamp) from cultured ICCs. An axopatch ID (Axon Instruments, Foster, CA, USA) was used to amplify membrane currents and potentials. The command pulse was applied using an IBM-compatible personal computer and pClamp software (version 6.1; Axon Instruments). Data

obtained were filtered at 5 kHz and displayed on an oscilloscope, a computer monitor, and using a pen recorder (Gould 2200, Gould, Valley View, OH, USA).

Results were analyzed using pClamp and Origin (version 6.0) software. All experiments were performed at $30\text{--}32^\circ\text{C}$.

Solutions and drugs

The physiological salt solution used to bathe cells (Na⁺-Tyrode) contained (mmol/L): KCl 5, NaCl 135, CaCl_2 2, glucose 10, MgCl_2 1.2 and HEPES 10, adjusted to pH 7.4 with NaOH. The pipette solution contained (mmol/L): KCl 140, MgCl_2 5, K_2ATP 2.7, NaGTP 0.1, creatine phosphate disodium 2.5, HEPES 5 and EGTA 0.1, adjusted to pH 7.2 with KOH.

Before the development of CGP37157, several benzodiazepines (except clonazepam) were used as mitochondrial Na⁺-Ca²⁺ exchange inhibitors^[12]. Clonazepam and CGP37157 were dissolved in dimethyl sulfoxide (DMSO) for 100 and 50 mmol/L stock solution, respectively and added (1 000 times dilution) to the bathing solution at the day of the experiment. The final concentration of DMSO in the bath solution was always $<0.1\%$, and we confirmed that this concentration of DMSO did not affect the results that were recorded. Nickel chloride was directly added to the pipette solutions at the day of the experiment. CGP37157 was purchased from Tocris Cookson (Ellisville, MO, USA). The rest of the drugs were obtained from Sigma (Sigma Chemical Co., USA), unless otherwise stated. Diltiazem was not used because of its known effects on the cell membrane Ca^{2+} channels.

RESULTS

Effect of clonazepam on the pacemaking activity of ICCs
Under a voltage clamp at a holding potential of -60 mV, clonazepam 50 $\mu\text{mol/L}$ inhibited the pacemaking currents of ICCs ($n=4$, Figure 1A). Clonazepam from 20 to 60 $\mu\text{mol/L}$ effectively inhibited Ca^{2+} efflux from mitochondria on the pacemaking activity of ICCs. Concentrations of clonazepam of >100 $\mu\text{mol/L}$ produced no further inhibition. The IC_{50} of clonazepam was 37.1 $\mu\text{mol/L}$ (Figure 1B).

Effect of CGP37157 on the pacemaking activity of ICCs

The benzodiazepine CGP37157 has been shown to be a more potent inhibitor than either clonazepam or diltiazem in terms of Ca^{2+} efflux measured in isolated mitochondria^[12]. Thus, CGP37157 was applied to examine its effect on the pacemaking activity of ICCs. Under a current clamp ($I=0$), CGP37157 was found to inhibit pacemaking potentials in a dose-dependent manner ($n=15$, Figures 2A-2C). CGP37157 from 10 to 30 $\mu\text{mol/L}$ effectively inhibited Ca^{2+} efflux from mitochondria on the pacemaking activity of ICCs. Concentrations of CGP37157 of >50 $\mu\text{mol/L}$ produced no further inhibition. The IC_{50} of CGP37157 was 18.2 $\mu\text{mol/L}$ (Figure 2D).

Effect of internal Ni^{2+} on the pacemaking activity of ICCs

Ni^{2+} is a competitive inhibitor of the Ca^{2+} carrier, but it is not transported into the mitochondria^[16]. Micromolar concentrations of nickel (Ni^{2+}) chloride have been reported

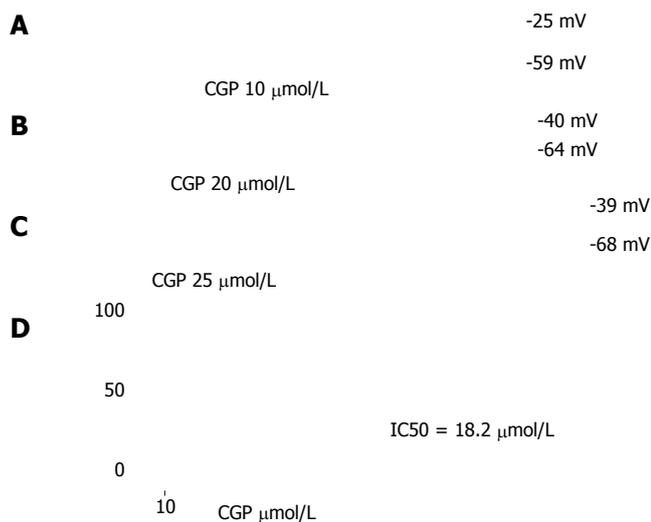


Figure 2 The effect of CGP37157 in the pacemaking activity of the ICCs. CGP37157 was applied to examine its effect on the pacemaking activity of ICCs. **A, B, and C:** Under a current clamp ($I=0$), CGP37157 inhibited the pacemaking potentials in a dose-dependent manner ($n=15$). **D:** CGP37157 from 10 to 30 $\mu\text{mol/L}$ effectively inhibited Ca^{2+} efflux from mitochondria in the pacemaking activity of ICCs. The IC_{50} of CGP37157 was 18.2 $\mu\text{mol/L}$.

to inhibit $\text{Na}^{+}\text{-Ca}^{2+}$ exchange in both vascular and non-vascular cells^[17]. In order to investigate the effect of NiCl_2 on the pacemaking activity of ICCs, we added 20 $\mu\text{mol/L}$ NiCl_2 to the internal solution. Under a voltage clamp mode at a holding potential of -60 mV, the pacemaking activity of ICCs showed a “wax and wane” phenomenon ($n=6$, Figure 3A). Also in current clamp mode ($I=0$), the same phenomenon was shown ($n=3$, Figure 3B). In case of 100 $\mu\text{mol/L}$ NiCl_2 , the pacemaking activity of ICCs stopped (data not shown).

DISCUSSION

Intracellular Ca^{2+} plays an important role in the regulation of various cellular functions including exocytosis, metabolic activity, contractile activity, and gene expression in excitable cells. In these cells, the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) is lowered by various mechanisms such as Ca^{2+} extrusion due to the actions of $\text{Na}^{+}\text{-Ca}^{2+}$ exchangers and Ca^{2+} pumps in the plasma membrane, Ca^{2+} uptake by Ca^{2+} pumps in the endoplasmic reticulum, and by Ca^{2+} uniporters in the mitochondria^[17]. Sequestered Ca^{2+} in mitochondria is, in turn, released to the cytoplasm via various mechanisms^[11]. $\text{Na}^{+}\text{-Ca}^{2+}$ exchangers and/or permeability transition pores are proposed to be involved in Ca^{2+} efflux from the mitochondria^[11].

In case of ICCs, pacemaking activity is associated with mitochondrial Ca^{2+} transients. Pacemaker currents and rhythmic mitochondrial Ca^{2+} uptake by ICCs are blocked by inhibitors of IP_3 -dependent Ca^{2+} release from the endoplasmic reticulum and by inhibitors of endoplasmic reticulum Ca^{2+} reuptake. Therefore, integrated Ca^{2+} management by endoplasmic reticulum and mitochondria is a prerequisite of electrical pacemaking in the gastrointestinal tract^[8].

CGP37157 is a benzodiazepine derivative that inhibits

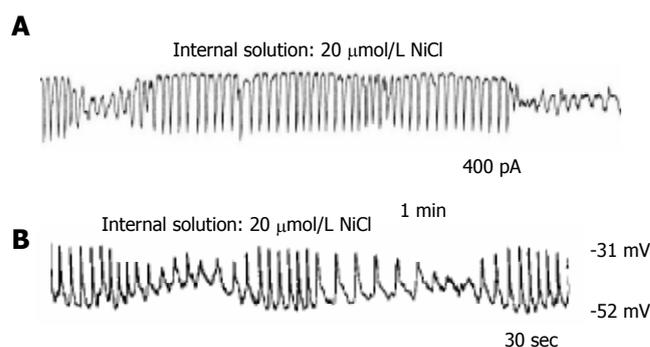


Figure 3 Effect of internal Ni^{2+} on the pacemaking activity of ICCs. In order to investigate the effect of NiCl_2 on the pacemaking activity of ICCs, we added 20 $\mu\text{mol/L}$ of NiCl_2 to the internal solution. **A:** Under a voltage clamp at a holding potential of -60 mV, the pacemaking activity of ICCs showed a “wax and wane” phenomenon ($n=6$). **B:** Current clamp mode ($I=0$) showed the same phenomenon ($n=3$).

electroneutral mitochondrial $\text{Na}^{+}\text{-Ca}^{2+}$ exchanger with sub-micromolar potency. In the heart, for example, this transporter is inhibited by CGP37157 at 400 nmol/L^[12,14]. Before the development of CGP37157, several related benzodiazepines (e.g., clonazepam and diltiazem) were used as mitochondrial $\text{Na}^{+}\text{-Ca}^{2+}$ exchange inhibitors^[14]. In general, few have reported that these compounds inhibit the cardiac plasmalemmal $\text{Na}^{+}\text{-Ca}^{2+}$ exchanger^[18,19]. Mitochondrial $\text{Na}^{+}\text{-Ca}^{2+}$ exchanger protein participates in Ca^{2+} efflux and operates in opposition to a Ca^{2+} uniporter within the inner mitochondrial membrane. Thus, the inhibition of mitochondrial $\text{Na}^{+}\text{-Ca}^{2+}$ exchanger leads to an increase in Ca^{2+} levels within the mitochondria^[14]. Calcium within the mitochondria serves as an important regulator of several key enzymes involved in energy metabolism. For example, the upregulation of the steady-state level of mitochondrial $[\text{Ca}^{2+}]$ ($[\text{Ca}^{2+}]_m$) result increases NADH production and stimulates oxidative phosphorylation^[14].

The benzodiazepine CGP37157 has been shown to be a more potent inhibitor than either clonazepam or diltiazem on Ca^{2+} efflux, as measured in isolated mitochondria^[12]. In ICCs, the IC_{50} s of clonazepam and CGP37157 were found to be 37.1 and 18.2 $\mu\text{mol/L}$, respectively. Thus, CGP37157 was about twofold more potent than clonazepam.

Ni^{2+} is a potent inhibitor of mitochondrial Ca^{2+} transport^[20] and a competitive inhibitor of Ca^{2+} carrier^[16]. Also micromolar concentrations of nickel (Ni^{2+}) chloride were found to inhibit $\text{Na}^{+}\text{-Ca}^{2+}$ exchanger in both vascular and non-vascular cells^[21]. Therefore, we have investigated the effect of NiCl_2 added to the internal solution. At 20 $\mu\text{mol/L}$, we observed a “wax and wane” phenomenon and at 100 $\mu\text{mol/L}$, the pacemaking activity of ICCs stopped.

Our data indicate that both clonazepam and CGP37157 inhibit the pacemaking activity of ICCs in a dose-dependent manner. The IC_{50} s of clonazepam and CGP37157 were 37.1 and 18.2 $\mu\text{mol/L}$, respectively. When 20 $\mu\text{mol/L}$ NiCl_2 was added to the internal solution, the pacemaking activity of ICCs showed a “wax and wane” phenomenon.

We conclude that mitochondrial $\text{Na}^{+}\text{-Ca}^{2+}$ exchange has an important role in intestinal pacemaking activity.

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RAPID COMMUNICATION

Combined resection and multi-agent adjuvant chemotherapy for desmoplastic small round cell tumor arising in the abdominal cavity: Report of a case

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Abstract

Desmoplastic small round cell tumor (DSRCT) is a rare, highly aggressive malignancy with distinctive histological features: a nesting pattern of cellular growth within dense desmoplastic stroma, occurring in young population with male predominance. The mean survival period is only about 1.5-2.5 years. The tumor has co-expressed epithelial, muscle, and neural markers in immunohistochemical studies. This work reports a 27-year-old man presenting with hematemesis and chronic constipation. Serial studies including endoscopy, upper gastrointestinal series, abdominal computed tomography and barium enema study showed disseminated involvement of visceral organs. The patient underwent aggressive surgery and received postoperative adjuvant chemotherapy consisting of 5-fluorouracil, cyclophosphamide, etoposide, doxorubicin, and cisplatin. He survived without any disease for 20 mo after the surgery. No standard treatment protocol has been established. Aggressive surgery combined with postoperative multi-agent adjuvant chemotherapy is justified not only to relieve symptoms but also to try to improve the outcome in this advanced DSRCT young patient.

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Key words: Desmoplastic small round cell tumor; Surgery; Chemotherapy

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INTRODUCTION

Desmoplastic small round cell tumor (DSRCT) is a rare malignancy with highly aggressive behavior spreading widely along the serosal surface. Gerald and Rosai^[1] first described the disease in 1989 in terms of distinctive pathologic findings: a nesting pattern of cellular growth within dense desmoplastic stroma, and immunohistochemical co-expression of epithelial, muscle and neural markers^[1-3]. A specific chromosome abnormality, t (11;22) (p13;q11 or q12) was identified^[4], and DSRCT is believed to be the result of involvement of both Wilms' tumor suppressor gene and Ewing's sarcoma gene located on Chromosome 11 and 22, respectively^[5]. The tumors usually present in young male individual, as a single mass or multiple masses in the abdominal-pelvic cavity with metastases common to peritoneum, liver and lymphoid tissue^[6]. Some cases had presentation sites in the scrotum^[6,7], pleural space^[6,8,9] and mediastinum^[6,10]. Complete resection is generally difficult to obtain due to multiple foci and dissemination. The standard treatment protocol has not been well established. Chemotherapy currently seems to be the best potential treatment. This work reports on a case of DSRCT with no disease for 20 months following complete resection of tumors and postoperative multi-agent adjuvant chemotherapy consisting of 5-fluorouracil (5-FU) and PAVEP (cyclophosphamide, etoposide, doxorubicin and cisplatin).

CASE REPORT

The 27-year-old man had intermittent epigastralgia, abdominal fullness, and chronic constipation for 3 months. He presented sudden onset of hematemesis (500 mL) outside our hospital on September 15, 2003. Physical examination revealed only mild epigastric tenderness. The hemoglobin level was normal. Endoscopy showed esophageal and cardiac ulcers without complete study owing to poor compliance of the patient. Upper gastrointestinal (GI) series demonstrated a mass with external compression at the gastric antrum (Figure 1). Abdominal computed tomography (CT) indicated a 5 cm heterogeneous mass located among the gastric antrum, duodenum loop and pancreatic head (Figure 2). Barium enema study showed external compression of the middle transverse colon, ileocecal valve and ascending colon (Figure 3). Laparoscopic biopsy was performed because multiple intra-abdominal organs were involved. A histo-

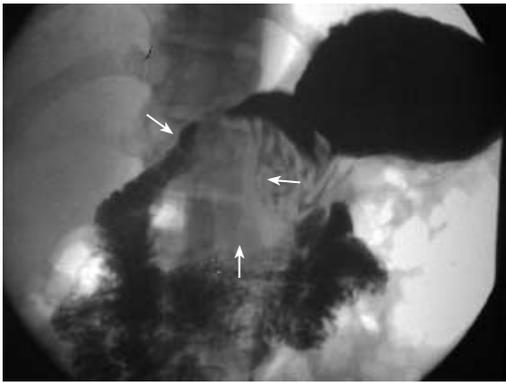


Figure 1 Upper gastrointestinal series shows that the gastric antrum (arrows) is deformed due to external compression.

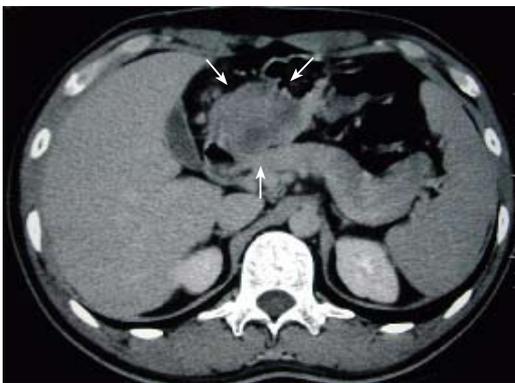


Figure 2 Abdominal computed tomography shows a 5 cm heterogeneous mass (arrows) located between the gastric antrum, duodenal loop, and pancreatic head.

pathologic exam of the specimen revealed poorly differentiated carcinoma. The patient was then referred to our hospital for further treatment.

No significant medical or pertinent family history was found. Additionally, no history of exposure to carcinogens such as radiation, tobacco or asbestos was identified. Hematogram showed hemoglobin, 110 g/L and white blood cells (WBC), $11 \times 10^9/L$. Biochemistry studies yielded normal results. Tumor markers such as carcinoembryonic antigen and carbohydrate antigen 19-9 were within the normal range. Laparotomy was performed on October 3, 2003 to relieve the patient's symptoms. During the operation, the gastric antrum, pancreas, terminal ileum and cecum, sigmoid colon, rectovesicular region and omentum were all involved by tumors. Right hydroureter was also identified due to external compression by the tumor. Subtotal gastrectomy, partial pancreatic resection, total omentectomy, proctocolectomy and J-pouch ileo-anal anastomosis were performed for complete resection of tumors. A double-J stenting was inserted through the ureterostomy by a urologist.

A histopathologic exam of the surgical specimens demonstrated invasive nests of poorly differentiated carcinoma, mainly involving omentum, pancreatic, and gastric, colonic and ileal muscular walls with focal mucosa extension. Large and small foci of tumor cells were observed in fibrous interstitium (Figure 4A). The tumor cells



Figure 3 Barium enema study demonstrates a mass compressing the middle transverse colon with tethering appearance, suggesting gastrocolic ligament invasion (black arrows). Long segmental encasement of cecum, proximal ascending colon (white arrows), and ileocecal valve (arrows head) is also evident.

had a small spherical or spheroid nucleus, which was rich in chromatin (Figure 4B). One of 20 peri-colic lymph nodes was positive for metastasis. The section margins were free of tumor. The immunohistochemical tests were positive for desmin (Figure 4C), epithelium membrane antigen and neuron-specific enolase, and focal positive for vimentin, indicating DSRCT.

J-pouch ileo-anal anastomosis leakage with intra-abdominal abscess occurred on postoperative day 7. The patient recovered after treatment by antibiotics and CT-guidance drainage. Postoperative adjuvant chemotherapy with high dose 5-FU (2600 mg/m^2 , day 1) was initiated on 24th October, 2003. The patient received altogether eight courses of chemotherapy of 5-FU thereafter. Another chemotherapeutic regimen consisting of PAVEP (cyclophosphamide 300 mg/m^2 , d 1-3; etoposide 75 mg/m^2 , day 1-3; doxorubicin 40 mg/m^2 , day 1; cisplatin 100 mg/m^2 , day 4) was administered on February 17, 2004. Neutropenic (WBC, $2 \times 10^9/L$) fever developed one week later even though prophylactic granulocyte colony-stimulating factor (G-CSF) was given the day following chemotherapy. The patient received broad-spectrum antibiotics treatment, and his fever was gradually controlled. He was discharged 12 d later when his WBC returned to normal. Another four chemotherapy courses with PAVEP regimens were completed on May 24, 2004. He survived without any disease 20 mo after the surgery.

DISCUSSION

DSRCT has a strong male predominance, and the majority arises below the diaphragm with serosal spreading and easily metastasizing to lung, liver, and lymphoid tissues^[2,6,11]. The most common site was the pelvis (62%), followed by spreading widely on the peritoneal surface (42%)^[12]. Symptoms such as abdominal pain (52.1%), increased abdominal girth (8.4%), and abdominal mass (5.6%) have been reported^[14]. Patients also presented with GI or genitourinary obstruction. GI bleeding does not appear to have been reported in the literature. This patient was the first case with DSRCT presented with GI bleeding. The bleeding was associated with tumors directly invading to the gastric mucosa.

The prognosis of DSRCT is dismal with a mean survival of 1.5-2.5 years since the wide spread of the tumor makes radical resection difficult to achieve, and

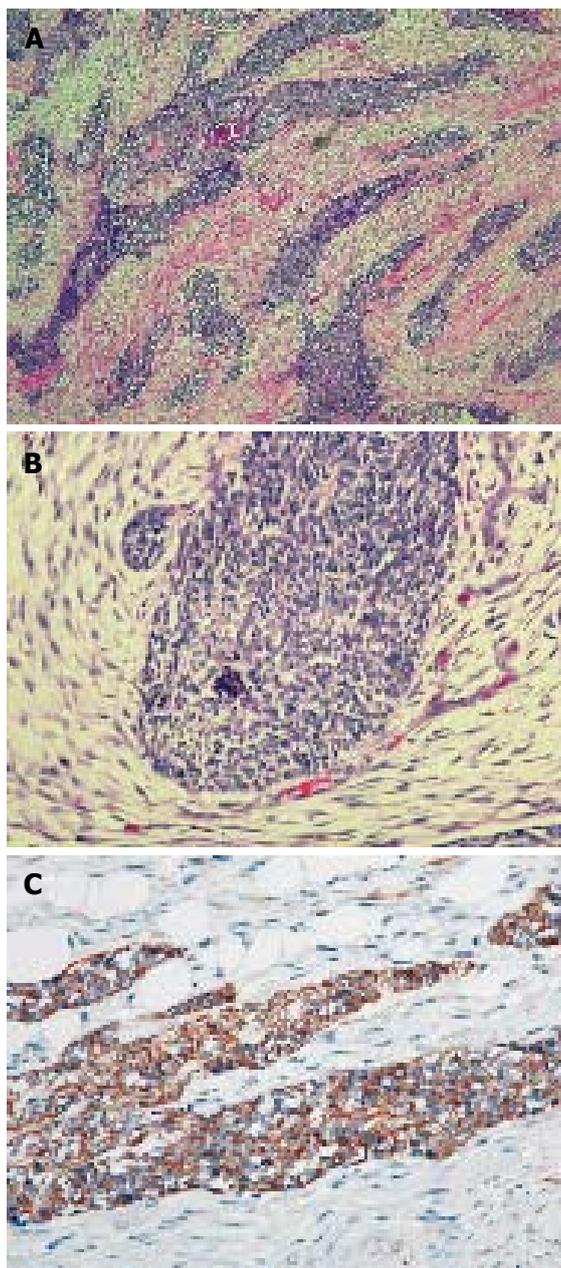


Figure 4A: Photomicrography depicts nests or clusters of small tumor cells outlined by characteristic desmoplastic stroma bands (hematoxylin and eosin staining, 100 \times). **B:** Photomicrography demonstrates the monomorphic, small round tumor cell population with a small spherical or spheroid nucleus, rich in chromatin (hematoxylin and eosin staining, 400 \times). **C:** The immunohistochemical staining of tumor cells is positive for desmin.

chemotherapeutic agents are only temporarily effective in treating this disease^[6,12-15]. Farhat *et al*^[13] recommended that PAVEP should be the first-line drug for treating DSRCT after reviewing 8 cases with complete remission from the treated 60 cases. However, grade four neutropenia occurred in 69% cycles while administering PAVEP regimens despite prophylactic use of G-CSF in 90% of cases in his report^[15]. Considering the severe and life-threatening side effects and uncertainty of therapeutic results of PAVEP regimens, other safer and effective chemotherapeutic agents should be considered prior to PAVEP.

5-FU is well-known as a chemotherapeutic agent and a radioactive sensitizer broadly used for GI tract cancers and

disseminated intra-abdominal metastatic adenocarcinoma. Gerald *et al*^[2] reported a successful application of 5-FU in treating intra-abdominal DSRCT. Kretschmar *et al*^[6] also reported 60% responsive rate to this tumor. Since DSRCT is a highly aggressive and progressive malignancy, post-operative adjuvant chemotherapy should be initiated as soon as possible. Post-operative anastomotic leakage and right urinary tract obstruction arose in our case. Therefore, 5-FU was applied instead of PAVEP regimen as the first-line drug to treat DSRCT considering the infection problems and the adverse effects of PAVEP regimens such as myelo-suppression and renal toxicity.

DSRCT is a disease so rare that no consistent response to chemotherapy was seen in the literature review^[12]. Kurre *et al*^[16] presented another alkylator-based chemotherapy protocol named P6 (cyclophosphamide, doxorubicin, vincristine, ifosfamide, and etoposide), which only improved the progression-free survival without significant survival benefit. Gil *et al*^[12] recommended performing perioperative intraperitoneal chemotherapy when treating DSRCT patients, but no evidence of prolonged survival was observed.

The effect of complete resection of disseminated tumors on survival is still unknown because of the rarity of achieving complete resection at operation. Gil *et al*^[12] reported that the median survival was 20 mo in 4 patients with complete resection of tumors compared with 11 mo in 3 cases without complete resection. Significantly, long-term survivors, including one patient who survived for 101 mo, have been reported when utilizing combined modality treatment comprising surgery and multi-agent chemotherapy. The influence of other salvage therapies, such as immunotherapy or bone marrow ablation, is still undetermined. In this work, the patient underwent complete resection of tumors and received postoperative adjuvant chemotherapy surviving without any disease and without gastrointestinal symptoms 20 mo after surgery, similar to that in Gil's report^[12].

In conclusion, the optimal treatment for DSRCT remains to be determined, indicating the rarity and virulent behavior of the disease. In this advanced DSRCT patient, aggressive surgery combined with postoperative systemic multi-agent adjuvant chemotherapy was justified not only to relieve his symptoms but also to try to improve his outcome. The effectiveness and safety of chemotherapeutic regimens still need to be improved to treat this disease as early as possible postoperatively, especially when patients cannot tolerate the toxicity of alkylator agents after such an aggressive surgery.

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CASE REPORT

Ruptured angiosarcoma of the liver treated by emergency catheter-directed embolization

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Abstract

Angiosarcoma is a rare primary malignant neoplasm of the liver with a poor prognosis. Here, we report a case of a patient with a ruptured hepatic angiosarcoma which was treated by emergency catheter-directed embolization, followed by left-sided hemihepatectomy.

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Key words: Ruptured angiosarcoma; Liver; Embolization

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INTRODUCTION

Angiosarcoma (AS) is a rare tumor entity which comprises less than 1% of all sarcomas^[1]. Although rarely observed, it is the third most common primary malignant tumor of the liver. The occurrence of AS in the liver shows a predilection for elderly males^[2]. The exposures to vinyl

chloride, arsenic, thorotrast and irradiation are associated with an increased risk for the development of AS of the liver and are considered as etiologic cofactors^[3-5]. Clinical diagnosis is usually hampered by the unspecific clinical signs and symptoms. Patients reported have been found with pain, loss of energy, and weight, hemorrhage and extrusion^[6,7,8]. The therapeutical options described are oncological liver resection and chemotherapy^[6,7,9]. Liver transplantation has not been reported to be significantly beneficial in this subgroup of sarcoma patients so far^[10]. The clinical course of AS, even after curative surgery and chemotherapy is aggressive with a poor prognosis has been reported^[7,11,12].

Here, we report about the rare case of a patient with acute bleeding to the abdominal cavity by a spontaneously ruptured AS of the liver.

CASE REPORT

A 76-year-old man was referred to the hospital with acute abdominal pain. With the onset of pain the patient collapsed. The subsequent consultation lead to emergency admission to the department of surgery. Apart from a slowly progressive local prostate cancer which was already resected transurethrally thrice (5 years ago, 1 year ago and palliatively 1 month before the admission), no significant disease was reported. The patient neither reported any kind of abdominal trauma nor prior abdominal surgery. Medication comprised antihypertensive drugs, iodine and androgene blockers since 1999. Besides that the patient had controlled his diabetes with an insulin pump.

At physical examination of the conscious patient, the abdomen was distended and showed a diffuse pain at deep palpation without clinical signs for peritonitis. Vital signs showed a slightly elevated blood pressure (140/70 mmHg) and a tachycardia (120 heart beats/min). Emergency blood test including hemoglobin, white blood count, electrolytes and hemostasis parameters revealed a reduced hemoglobin with 6.9 g/dL. Besides that, infection parameters were elevated with leukocytes of 16.0/nL and a C-reactive protein of 75.4 mg/L. Cholinesterase and albumin were also lowered significantly. Hemostasis parameters were normal.

The ultrasound examination of the abdomen, performed as the primary diagnostic modality, showed free abdominal fluid and a pathological finding in the left lobe of the liver, diagnosed as a ruptured liver lesion, at that time suspicious of a liver cell adenoma due to a 5-year



Figure 1 CT scan before embolization.

period of regular intake of androgene blockers. In order to identify the location of the active bleeding within the liver, an additive contrast enhanced computed tomography (CT) of the abdomen was carried out. The CT images confirmed a ruptured lesion of the left liver lobe with an ongoing active arterial bleeding located centrally within segments 2 and 3. In addition, a considerable amount of intra-abdominal blood (1-2 L) was diagnosed (Figure 1). The patient received blood transfusions (2 erythrocyte concentrates) and stayed hemodynamically stable. The interdisciplinary decision between radiologists and surgeons was made for therapeutic radiological intervention.

Subsequently, the patient was transferred to the radiological catheter room. The intervention was carried out by puncture of the right femoral artery and selective catheterization of the left hepatic artery. After cannulation of the left liver artery, the left lobe of the liver was completely and successfully embolized with 2 mL of embosphere 500-700 and 2 mL of embosphere 900-1 200. After intervention, there were no more signs for active bleeding, and blood pressure and heart rate stayed regular (Figure 2A, before embolization and Figure 2B after embolization). The patient was treated post-interventionally for 2 d at the intensive care unit before he was referred in a completely stable condition to the regular ward. Consecutively, elective surgery was scheduled to treat the abdominal hematoma and the ruptured lesion of the left lobe of the liver.

Preoperatively performed endoscopy of the upper GI tract and colonoscopy showed no substantial pathological findings (small axial hiatus hernia and a diverticulosis of the sigma without signs for inflammation).

Intraoperative findings

Surgery was performed 7 d after embolization. Explorative laparotomy revealed approximately 3 L of intra-abdominal hematoma without signs for active liver bleeding. Exploration of the left lobe of the liver revealed a ruptured hepatic tumor to the peritoneal cavity with a diameter of 12.5 cm which showed macroscopic characteristics of a hepatic adenoma. In order to treat the ruptured left lobe and to remove the liver tumor,

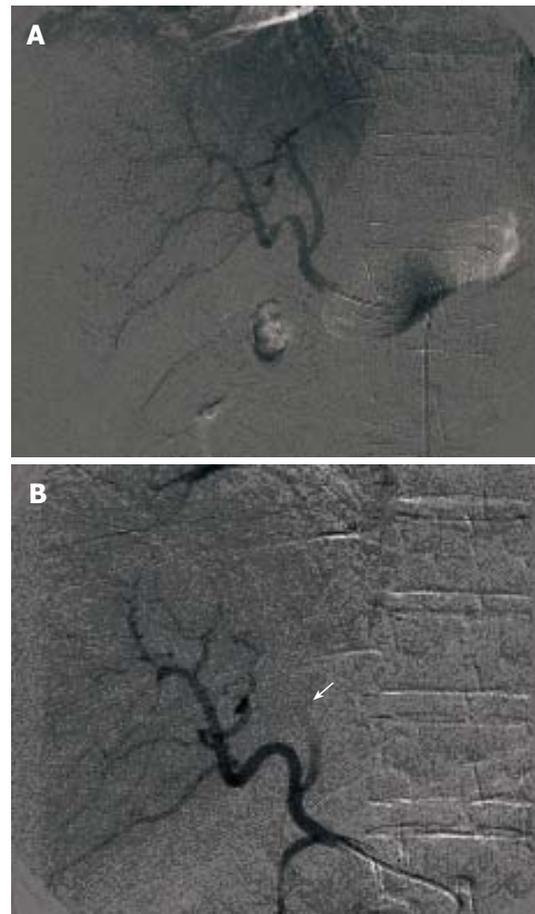


Figure 2 Angiography **A**: before embolization **B**: after embolization.

a left sided hemihepatectomy was carried out. After resection, intraoperative sonography of the remaining liver parenchyma showed no pathological findings. Intraoperatively and during the early post-operative course, the patient received five erythrocyte-concentrates and two units of fresh frozen plasma. The post-operative course was uneventful and the patient could be discharged from hospital 19 d after the surgery.

Histopathologic findings

Histopathological examination of the specimen revealed a ruptured hematous hepatic malignancy of 12.5 cm diameter without sharp demarcation. The histopathological examination and the immunostaining diagnosed a tumor with endothelial proliferates along the liver sinusoids, large necroses and cell atypias consisting of solid and papillary tumor parts. Immunohistochemical staining for CD31, CD34, MIB-1 confirmed the diagnosis of a hemangiosarcoma. The final diagnosis was a ruptured AS of the left lobe of the liver with tumor-free resection margins at microscopic evaluation.

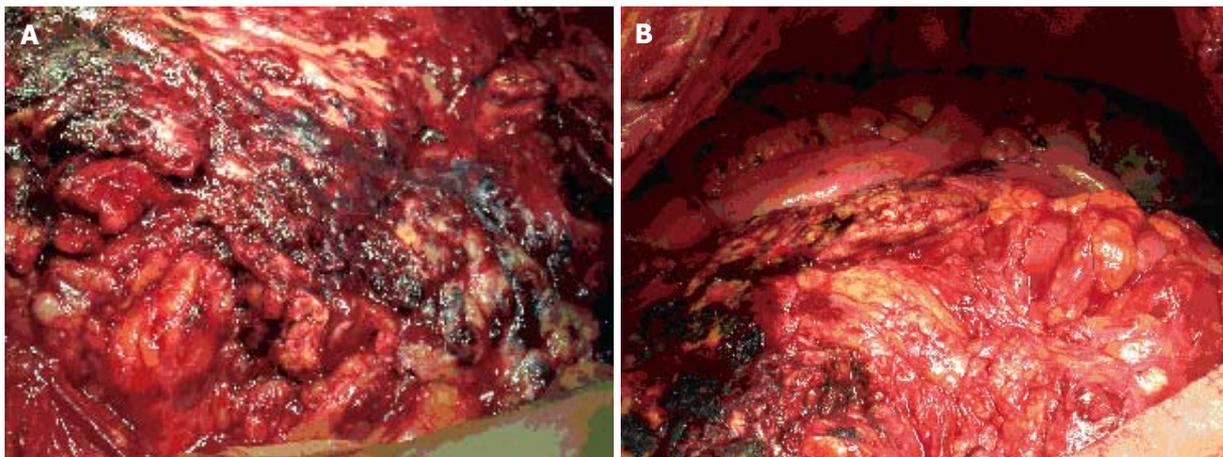
Postoperative course

Four weeks after primary surgery, the patient was readmitted to the hospital with the suspicion of recurrent acute intra-abdominal bleeding. A CT-scan showed free intra-abdominal fluid collection. An angiography showed small contracted vessels of the liver without proof of

Table 1 Case reports focusing on patients with hepatic AS undergoing surgery and/or embolization from 1995 to 2005

| Reference | Year ¹ | Number ² | Age (yr) | Sex (M/F) | Clinical symptoms | Surgery | Resection margin | Additive Therapy | Metastasis at presentation | Emergency | Survival |
|--|-------------------|---------------------|----------|-----------|--|----------------------------------|-------------------|----------------------------------|----------------------------|---------------------|------------------------------|
| Tordjman <i>et al</i> ^[27] | 1995 | 1 | 34 | M | ? | No | | Chemo-embolization | No | Yes | 15 mo |
| Timaran <i>et al</i> ^[29] | 2000 | 1 | 47 | F | Right upper quadrant discomfort | Right hepatic lobectomy | Negative | No | No | No | Alive (120 mo postoperative) |
| Kirschstein <i>et al</i> ^[30] | 2000 | 1 | 73 | F | Abdominal pain | Right hepatectomy | Positive | No | Yes | No | 6 wk |
| Hoppe <i>et al</i> ^[26] | 2001 | 1 | 72 | F | Pain, weight loss, hemodynamic instability | Liver segment resection | ? | Preoperative embolization | No | Spontaneous rupture | 2 wk |
| Ozden <i>et al</i> ^[10] | 2003 | 1 | 54 | F | ? | Right hepatectomy + relaparotomy | Positive/negative | Postoperative chemo-embolization | No | No | 64 mo |
| Horger and Fendel ^[25] | 2003 | 1 | 69 | F | Right upper quadrant pain, bleeding | Right hepatectomy | ? | Preoperative embolization | No | Spontaneous rupture | ? |
| Almogly <i>et al</i> ^[8] | 2004 | 2 | 33 | F | Bleeding, right upper quadrant pain | Right hepatectomy | ? | No | No | Yes | Died intra-operatively |
| | | | 68 | M | Bleeding | Right hepatectomy | Positive | Post-operative chemotherapy | No | Yes | 7 mo |

¹Year of publication; ²number of patients.



Figures 3 A and B Intraoperative pictures of peritoneal sarcomatosis.

active bleeding. The patient showed signs of hemorrhagic shock and received four erythrocyte concentrates and six units of fresh frozen plasma. Duplex-sonography showed reduced perfusion of the liver. Liver transaminases were elevated and the patient suffered simultaneously from a non-STEMI myocardial infarction without indication for acute catheter intervention.

In the following course the patient's abdomen became more and more distended with an aggravated pulmonary situation and progredient amounts of free intra-abdominal fluid. At this point, indication for explorative laparotomy was stated. Intraoperatively, multiple bleeding AS were found in the liver with massive peritoneal sarcomatosis (Figures 3A and 3B). Hemorrhage control of the diffuse bleeding could not be obtained surgically. The abdomen was closed and the patient was taken to the intensive care unit at basal infusion and kept on sedation and mechanical

respiration. Few hours after the explorative laparotomy the patient died from persistent intra-abdominal bleeding from the angiosarcomatosis lesions.

DISCUSSION

Among malignant vascular tumors, AS is one of the most common subtypes diagnosed in patients undergoing surgery^[13]. AS has a predilection for cutaneous sites, mostly in the head and neck region of elderly male patients^[14]. AS also affects gastrointestinal organs^[9,15-18] and hepatic AS is the second most common primary malignant neoplasm of the liver^[19]. However, there have only been a few case reports available in the literature concerning primary hepatic AS over the past 10 years (Table 1).

The clinical course of AS is aggressive and prognosis is poor. In a study of 67 patients with AS, an overall 5-year survival of 35% has been reported^[20]. Morgan *et al*^[21] investigated 47 patients with cutaneous AS, outlining the

restricted prognosis and demonstrating a 5-year survival of 34%. Another analysis showed a median overall survival of 28.4 months in a cohort of 88 patients with AS of the scalp^[14]. We observed a lethality of 65% in 43 patients who underwent surgery for AS with a median follow-up of 56 months^[13].

AS presents with various unspecific clinical symptoms and a multilocular occurrence. Different sites of occurrence go along with a wide range of non-specific symptoms such as pain, swelling, extrusion, hemorrhage, or bowel obstruction^[22]. Tumors of the skin and of superficial or deep soft tissue are usually biopsied preoperatively and thus, therapeutic decisions are made before surgery^[13]. However, as also reported here, organotropic AS may be diagnosed consecutive to emergency treatment^[23-26] or after the surgery for assumed benign disease. Thus, AS is often diagnosed unexpectedly after surgical procedures which do not meet oncological treatment standards.

To our knowledge, there are only two cases of hepatic AS reported to date, which have been treated by emergency catheter-directed embolization with consecutive liver resection (Table 1)^[24,25]. Catheter-directed intervention is a useful method to cease acute arterial bleeding from the liver. The most common indication is traumatic laceration of the liver parenchyma^[27]. Spontaneous bleeding and the formation of hematoma impede the clinical differentiation of the cause of the hemorrhage. In benign lesions (adenoma), a subsequent surgical resection of the affected parenchyma is curative. The catheter-directed embolization allows to perform a planned and well structured operation without the need of emergency liver surgery with high morbidity and lethality. In emergency cases with spontaneous bleeding, a differentiation of malignant or benign disease is impossible. Therefore, the final diagnosis is usually made by the histopathological evaluation of the resected specimen. At the time of the histopathological diagnosis, the prognosis of the disease becomes clear. In ruptured AS of the liver, the prognosis is devastating. Rupture in these cases leads to tumor cell spillage and consecutive diffuse angiosarcomatosis in the peritoneum. The angiosarcomatous lesions consecutively induce diffuse hemorrhage with subsequent loss of large amounts of blood. The acute bleeding from diffuse peritoneal angiosarcomatosis cannot be cured by surgical intervention and thus seems to be the leading cause for death in this rare subset of patients with AS. In an own observational series of 22 AS, 6 primary hepatic AS were found. Three of these patients died within 4 months and one patient 29 months after the diagnosis in spite of surgical intervention. The preliminary cause of death in the latter patients was peritoneal sarcomatosis with diffuse intra-abdominal bleeding^[13].

The clinical course of the patient reported here shows that there is no effective treatment available till date either for cure or palliation of ruptured AS of the liver. The value of chemotherapy applied to the peritoneal cavity or systemically has not been clarified nor the value of experimental antitumoral strategies such as molecular therapies (e.g. with VEGF-antagonists) or hyperthermia. This clinical situation underlines the need for new therapy

modalities and broader based clinical studies in patients with ruptured AS of the liver. An effort needs to be undertaken to include patients with ruptured AS of the liver after surgery into prospective trials. The opportunity to establish an international study group may, furthermore, allow a collection of tumor samples from AS in a reasonable number. Consecutive molecular analysis could help to establish targeted antitumoral therapies for these rare and extremely lethal malignancies.

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First report of a *de novo* germline mutation in the *MLH1* gene

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Abstract

Hereditary non-polyposis colorectal carcinoma (HNPCC) is an autosomal dominant disorder associated with colorectal and endometrial cancer and a range of other tumor types. Germline mutations in the DNA mismatch repair (MMR) genes, particularly *MLH1*, *MSH2*, and *MSH6*, underlie this disorder. The vast majority of these HNPCC-associated mutations have been proven, or assumed, given the family history of cancer, to be transmitted through several generations. To the best of our knowledge, only a single case of a *de novo* germline MMR gene mutation (in *MSH2*) has been reported till now. Here, we report a patient with a *de novo* mutation in *MLH1*. We identified a *MLH1* Q701X truncating mutation in the blood lymphocytes of a male who had been diagnosed with rectal cancer at the age of 35. His family history of cancer was negative for the first- and second-degree relatives. The mutation could not be detected in the patient's parents and sibling and paternity was confirmed with a set of highly polymorphic markers. Non-penetrance and small family size is the common explanation of verified negative family histories of cancer in patients with a germline MMR gene mutation. However, in addition to some cases explained by non-paternity, *de novo* germline mutations should be considered as a possible explanation as well. As guidelines that stress not to restrict MMR gene mutation testing to patients with a positive family history are more widely introduced, more cases of *de novo* MMR gene germline mutations may be revealed.

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Key words: *MLH1*; *de novo*; HNPCC; Germline mutation

Stulp RP, Vos YJ, Mol B, Karrenbeld A, de Raad M, van der

INTRODUCTION

Hereditary non-polyposis colorectal carcinoma (HNPCC) is a genetic disorder caused by germline mutations in DNA mismatch repair (MMR) genes, especially *MLH1*, *MSH2* and *MSH6*. HNPCC is characterized clinically by the early-onset colorectal and endometrial cancer as well as a range of other tumor types. In contrast to familial adenomatous polyposis (FAP), HNPCC lacks an easily recognizable clinical phenotype in individual patients. The original and revised Amsterdam criteria (AC and AC II) introduced to clinically define HNPCC therefore include specific characteristics of the family history that should be met^[1,2]. However, it has become clear that HNPCC patients may have a negative family history. Small family sizes and less than 100% penetrance of the germline mutations involved are its most common explanation. Here, we report the rare phenomenon of a *de novo* germline MMR gene mutation as the cause of a negative family history in a patient with hereditary non-polyposis colorectal carcinoma.

CASE REPORT

A 35-year-old male had been referred to our department for genetic testing and counseling because he had very recently been diagnosed with a T₄N₀M₀ rectal adenocarcinoma. Family history of cancer was negative for the first- and second-degree relatives of the patient. The patient's mother, father, and sibling (aged 60, 61, and 38 years, respectively) were healthy.

As part of an ongoing study on the genetics of colorectal cancer, testing for tumor microsatellite instability (MSI), tumor immunohistochemistry for the *MLH1*, *MSH2* and *MSH6* proteins as well as mutation analysis of *MLH1*, *MSH2* and *MSH6* are offered to all the patients with colorectal cancer diagnosed before the age of 50 who are referred to our department for genetic testing and counseling.

DNA was extracted from formalin-fixed and paraffin-embedded tumor and non-tumor tissues as described previously^[3]. Subsequent MSI analysis was performed using criteria and primers as described in the Bethesda

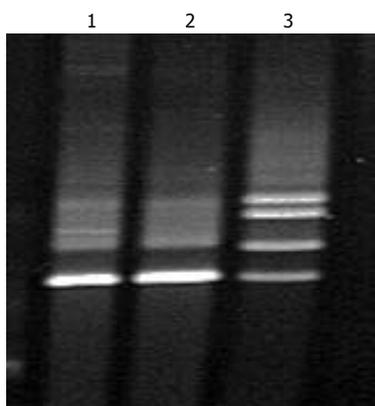


Figure 1 Denaturing gradient gel electrophoresis (DGGE) analysis of exon 18 of *MLH1* gene. Lanes 1 and 2: Father and mother, respectively; lane 3: index patient.

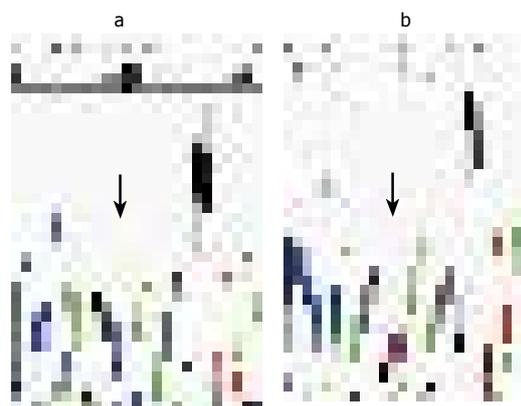


Figure 2 DNA sequence analysis of exon 18 of *MLH1* gene. (a) A control sample without mutation. (b) the index patient showing a C to T substitution of base 2101 leading to Gln701Stop.

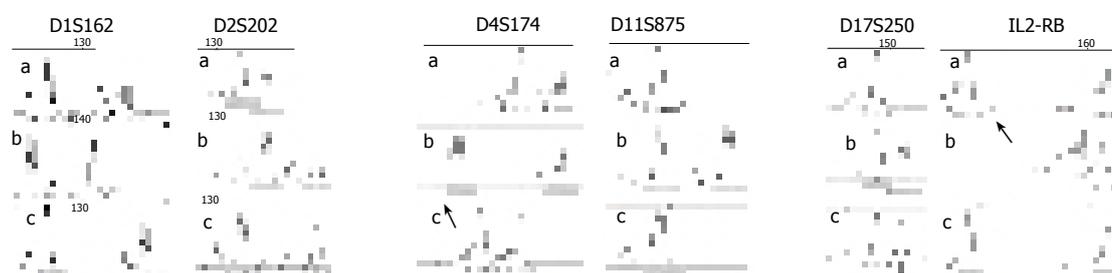


Figure 3 Paternity test using six highly informative microsatellites. a: The index patient; b: his father; c: his mother.

guidelines^[4]. PCR products were analyzed on 60 g/L denaturing polyacrylamide gels with an LKB ALF DNA sequencer. For data analysis, the DNA fragment analyzer (Pharmacia) was used.

Immunohistochemistry for *MLH1*, *MSH2*, and *MSH6* protein expression was carried out as described previously^[5]. Protein expression in normal tissue surrounding the cancer served as internal control.

Mutation analysis of the *MLH1* and *MSH2* genes in DNA obtained from peripheral blood lymphocytes was carried out using denaturing gradient gel electrophoresis (DGGE), followed in case of aberrant patterns by direct sequencing of independently amplified PCR products as previously described^[6,7]. To investigate paternity, a panel of six highly polymorphic markers was analyzed with PCR using markers D1S162, D2S202, D4S174, D11S875, D17S250, and IL2-RB in the patient and his parents. PCR products were analyzed on 60 g/L denaturing polyacrylamide gels with an LKB ALF DNA sequencer. For data analysis, the DNA fragment analyzer (Pharmacia) was used.

The tumor of the patient revealed microsatellite instability (MSI-H), as well as loss of immunohistochemical staining for the *MLH1* protein with a positive internal control. Mutation analysis revealed a C to T substitution at nucleotide 2101 in exon 18 of the *MLH1* gene, leading to an early stop codon (Gln701Stop). Neither the father nor the mother carried the mutation identified in their son (Figures 1 and 2). All six tested markers were in concordance with paternity (likelihood: >99.9%) (Figure 3)

DISCUSSION

Although the Q701X *MLH1* mutation has not been

reported before in either the Human Gene Mutation Database (HGMD) and the ICG-HNPCC database, we have no reason to doubt its pathogenicity given its truncating nature and the findings of MSI-H and loss of staining for the *MLH1* protein in the patient's tumor^[8,9].

De novo germline mutations of the MMR genes in HNPCC are rare. To the best of our knowledge, only one single case, involving *MSH2*, has been reported so far^[10]. We have identified a *de novo* germline mutation in the *MLH1* gene. The classification "germline" mutation is generally used in oncogenetics to distinguish mutations that can be transmitted to the offspring from the somatic mutations occurring locally in tissues and which may lead to local tumor development. For practical purposes, we therefore used the "germline" label. Strictly speaking, mutations classified as *de novo* germline mutations should refer to a single mutation occurring in a single germcell. Clinically, these often cannot be distinguished from an early postzygotic mutational event in the patient, or from germline mosaicism or somatic mosaicism extending to extra-gonadal tissues in the parents. We did not have the opportunity to test multiple tissues in our patient or his parents for the mutation. The patient's single sibling was offered mutation analysis and was shown to carry the wild-type *MLH1* alleles. Therefore, we lack proof of the germline nature of the *de novo* mutation reported by us and the same is true for the *MSH2* case published earlier by Kraus *et al*^[10]. In clinical practice, this lack of proof does not lead to any obvious problems. One will simply discuss with the patient the fact that his or her siblings have a, probably small, risk to carry the mutation as well and one will offer to test them for it.

Non-penetrance and small family size is the common explanation of verified negative family histories of cancer

in patients with a germline MMR gene mutation. However, in addition to some cases due to non-paternity, *de novo* “germline” mutations should be considered as a possible explanation as well. The percentage of these mutations in HNPCC remains to be established. As guidelines (e.g., the Bethesda guidelines)^[4] that stress not to limit MMR gene mutation testing to patients with a positive family history are more widely introduced, more cases of *de novo* “germline” MMR mutations are yet to be revealed.

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CASE REPORT

Successful percutaneous drainage of a giant hydatid cyst in the liver

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CASE REPORT

A 35-year-old female was hospitalized for abdominal pain, bloating, and vomiting. Plain X-ray pictures exhibited bowel obstruction for which she underwent emergency surgery. On the second postoperative day, she started to complain about dull right upper quadrant abdominal pain and bloating. Abdominal CT scans revealed very large cystic lesion with an inhomogeneous fluid content. The cyst occupied the whole right lobe of the liver compressing the surrounding organs and dislocating the left lobe of the liver towards the left hypochondrium (Figure 1). Additionally, several more cysts of various sizes were seen in the whole abdomen. This radio-morphology raised the possibility of a hydatid disease (caused by ruptured hydatid cyst) confirmed through positive echinococcus IgG serology (ELISA). Interestingly, no hypereosinophilia could be detected through repeated blood tests.

Mebendazole (200 mg/d for 3 d) was administered in order to prevent anaphylactic reactions and the dissemination of the disease. After obtaining written consent from the patient, the site of the puncture was defined to the X–XI intercostal space. The procedure was performed under ultrasound guidance (Diasonics Master

Series VST) and the patient was closely monitored for any sign of anaphylaxis. The cyst that was punctured on its free surface with an 18 gauge needle contained a dense whitish fluid. Samples were taken for parasitological examinations. Because of the huge size of the cyst, an 8F pigtail catheter (polyurethane drainage catheter, COOK) with 32 side holes was introduced into the cyst. After aspiration of 6 200 mL of the cystic fluid (Figure 2), 150 mL of 15% hypertonic saline was injected into the cyst cavity. Twenty minutes later, the catheter was attached to a vacuum tube and left for suction drainage. This cycle (hypertonic 15% saline injection and 20 min later reaspiration) was repeated daily with 40 mL 15% sterile saline. The initial procedure lasted for 30 min. At the end of the flushing series, absolute alcohol of 30 mL was injected and removed after 20 min during the last 5 days. During the procedure the patient remained symptomless and no anaphylactic reaction could be observed. Ultrasonographically, the cyst decreased in size gradually with only a 7.6 mm subcapsular fluid collection remaining under the right diaphragm. The total time of percutaneous drainage took 43 d.

Under light microscopy, the aspirated cystic fluid showed clear evidence of hydatid scolices verifying the presumed diagnosis of echinococcosis. After 6 weeks of percutaneous drainage the catheter was removed. Abdominal CT scans exhibited total disappearance of the cyst. Subsequently, the patient was discharged and reported no symptom relapses on the follow-up period of 1 year.

DISCUSSION

Echinococcus granulosus is a small, 5-mm-long tapeworm being responsible for unilocular hydatid cyst disease. Human infection by *E. granulosus* occurs most commonly in sheep- and cattle-raising areas where dogs assist in herding; the infection is more frequent in Eastern Europe, the Mediterranean, Australia, New Zealand, Chile, Argentina, and Africa. Human beings are usually infected as intermediate hosts when they ingest egg-contaminated food or water. Over 50% of all human *E. granulosus* infections involve the liver^[1]. Additional common sites for hydatid cysts are the lungs, spleen, kidneys, heart, bones, and brain^[2].

Since hydatid cysts grow at a rate of about 1–5 cm/year, a long latent period is typical. The symptoms of hydatid disease are related primarily to the mass effect of the slowly enlarging cyst: right upper quadrant pain from the stretching hepatic capsule, jaundice from compression

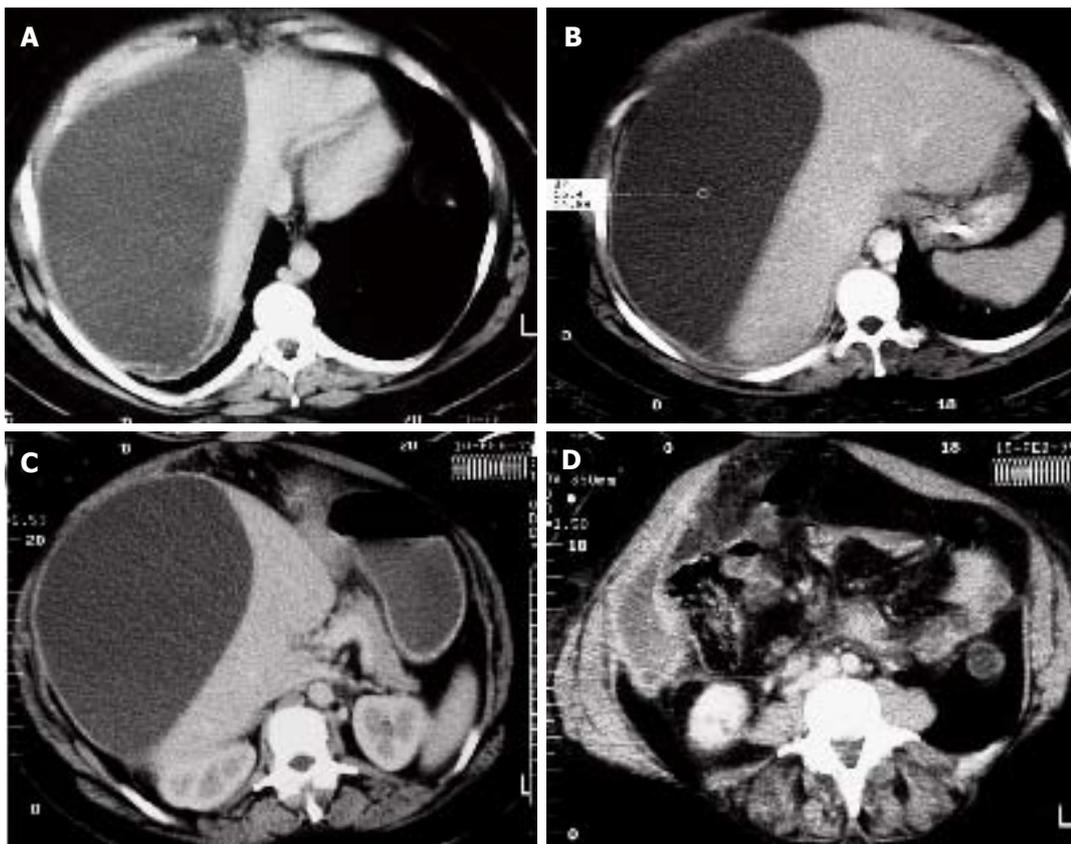


Figure 1 Abdominal CT scan. **A:** Axial CT scan shows the large inhomogeneous cystic fluid collection occupying the whole right lobe of the liver; **B:** CT scan of the cystic lesion on the level of spleen; **C:** the largest cystic lesion compresses the left lobe of the liver dislocating the nearby organs; **D:** cystic collection was seen also in the pararenal spaces and extended into the small pelvis.



Figure 2 **A:** The aspirated fluid (6 200 mL); **B:** drainage catheter with sucking bottle.

of the bile duct, or portal hypertension from portal vein obstruction. Following a presumably long latency, our patient presented with symptoms related to bowel obstruction caused by the space-occupying effect of the giant hydatid cyst. CT scans and ultrasonography are very useful for revealing well-defined cysts with thick or thin walls which correspond to our case as well^[2]. The 6.2-L large cyst of our case is the largest hydatid cyst published in the literature. The visualization of daughter cysts within the larger cyst and the mural calcification helps to distinguish *E. granulosus* infection from carcinomas, bacterial or amebic liver abscesses, or hemangiomas^[3]. Either spontaneously or as a result of a complication to surgery, free peritoneal rupture of the cyst can lead to fatal anaphylaxis. On the long term, leakage of the cyst may cause an intense antigenic response, resulting in eosinophilia, bronchial spasm, or anaphylactic shock.

Open surgical drainage of the hydatid cysts has been the preferred method of therapy. Several surgical techniques have been applied for the treatment of hydatid disease: simple-closure tube drainage, marsupialization, cystectomy, total pericystectomy, hepatic resection, and cavity management (omentoplasty, internal drainage)^[4]. Among them, total pericystectomy was found to be the most effective method at reducing recurrence and improving resolution of the hepatic defect. The mortality rate after surgery ranges from 0% to 6.3%, while the rate of postoperative complications varies between 12.5% and 80%^[4]. Jabbour *et al.*^[5] suggested that surgery should be the treatment of choice for large, multiple, complicated, and recurrent hydatid cysts of the liver.

The percutaneous treatment has been used in many hospitals as the alternative method to surgery^[6]. A prospective randomized trial of 50 patients found that

percutaneous drainage and hypertonic saline irrigation of the cysts combined with albendazole is as effective as surgical drainage, with fewer complications and shorter durations of hospitalization^[7]. A long-term follow-up study has also confirmed the effectiveness of percutaneous drainage with mortality and morbidity rates more favorably than those of conventional surgical approaches^[8]. The generally accepted and safe technique for percutaneous drainage is called PAIR that stands for puncture, aspiration of cyst, injection of hypertonic saline and absolute alcohol, and reaspiration^[9]. Hypertonic saline exerts a scolical effect and prevents the formation of daughter cysts through the irrigation of the germinative layer^[10]. Absolute alcohol is the agent of sclerotherapy against the wall of the hydatid cyst. Formerly, the percutaneous drainage of the hydatid cyst was deemed to be a hazardous procedure due to the potential risk of anaphylaxis and dissemination^[11]. Filice and Brunetti^[12], however, observed no anaphylactic reaction or peritoneal dissemination having applied the PAIR technique for 231 cysts in 163 patients. In agreement with this report, Haddad *et al*^[13] experienced only minor complications including pain, mild fever, pleural effusion, and transient hypernatremia in their series. In a 6-9 year follow-up involving ultrasonography, CT scans, and serology tests, no local recurrence or spread of the disease were found^[13]. Pelaez *et al*^[14] successfully treated 55 hydatid cysts in the liver in 34 patients with the combination of PAIR technique and albendazole prophylaxis. They reported only urticaria and subcapsular hematoma as minor complications and the mean hospitalization duration was 1.82 d. Akhan *et al*^[4,8] reported good accuracy rate and low complication rate in the percutaneous treatment of hydatid disease. Using the percutaneous drainage treatment combined with mebendazole prophylaxis in our patient, we could successfully manage the largest hydatid cyst ever reported. The applied drainage method seems to be useful for the treatment of huge hydatid cysts and has also been recommended as an effective and safe therapeutic choice.

In summary, this patient has demonstrated that percutaneous drainage is the appropriate and feasible alternative to surgery in treating hydatid cysts even of this

giant size with a low risk of severe complications.

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Small gastrointestinal stromal tumor concomitant with early gastric cancer: A case report

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Abstract

The term gastrointestinal stromal tumors (GISTs) is defined diagnostically as the main group of mesenchymal tumors with spindle or epithelioid cells arising from the wall of the gastrointestinal tract with immunohistochemical reactivity for CD117 antibody. Previous studies revealed that cells in GISTs express a growth factor receptor with tyrosine kinase activity (termed c-kit), which is the product of the c-kit proto-oncogene. The most specific and practical diagnostic criteria for GISTs are: immunohistochemically determined c-kit (CD117) expression; mitotic score; and tumor size. A small GIST concomitant with early gastric cancer is rarely encountered clinically. Herein we have reported a case of a 1.1-cm GIST detected by esophagogastroduodenoscopy concomitant with a IIC type of early gastric cancer (signet ring cell type). It was detected during a routine physical health examination. To our knowledge, this is the first report of a small GIST concomitant with a signet ring cell type of early gastric cancer.

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Key words: Gastrointestinal stromal tumor; Early gastric cancer; *H. pylori* infection; Biopsy urease test; CD117

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INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are malignant or potentially malignant tumors and are considered to have a specific molecular pathogenesis. They are

distinguished from other mesenchymal tumors by optimal immunostaining for CD117 and a prognostic classification is based on tumor size, mitotic score, and MIB-1 grade^[1]. Gain-of-function mutations of the *c-kit* gene and immunoreactivity of the c-kit protein (CD117) in many GISTs support the idea that GISTs are a biologically distinct entity. We have reported a case of a small GIST concomitant with a IIC type of early gastric cancer (signet ring cell type) and provided a literature review.

CASE REPORT

A 70-year-old yellow race female came to our hospital for a physical health examination in November 2003. She had no past history of epigastralgia or peptic ulcer. Esophagogastroduodenoscopy (EGD) showed one approximately 0.4-cm sessile polyp with a smooth surface at the right upper posterior wall of the gastric fundus (Figure 1A). Biopsy was taken and three specimens were acquired. Histologically, one of the three specimens showed whorling bundles of spindle cells with mitosis. Only chronic gastritis was found in the other two specimens. A GIST was suspected. EGD simultaneously detected one approximately 1.7 cm × 1.4 cm, depressed, flat white-red based lesion with oozing hemorrhages at the gastric angle. The lesion looked like a IIC type of early gastric cancer (Figure 1B). Biopsy was carried out and 13 specimens were obtained. Pathological sections demonstrated an ill-defined tumor with signet ring cells within the gastric mucosa. A poorly differentiated adenocarcinoma was diagnosed and *Helicobacter* microorganisms were found. An imaging study with abdominal computed tomography demonstrated no remarkable mass lesion at the posterior wall of the gastric fundus and gastric angle. There was no fluid collection, and no mass lesion or lymph node was found in the intraperitoneal cavity, liver, kidneys, and other organs. Owing to its malignant nature, surgical intervention was performed one week later. Grossly, we observed a 1.1 cm × 0.8 cm × 0.7 cm fundal mass protruding from the inner muscularis propria to the mucosa (Figures 2A and 2B). Histologically, the gastric fundal tumor demonstrated whorling sheets of spindle cells which stained positively for CD117, CD34, NSE, S-100 protein, and actin-851 antibodies after immunohistochemical (IHC) staining (Figures 2C and 2D). GIST, with combined smooth muscle-neural differentiation, was diagnosed. The gastric angular tumor showed a residual adenocarcinoma of the signet ring cell type within the mucosa (Figures 2E and 2H). The postoperative period of the patient was uneventful, and

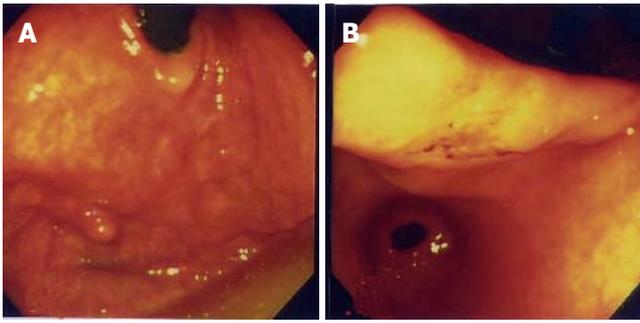


Figure 1 Esophagogastroduodenoscopic examination. **A:** A 0.4-cm sessile polyp with a smooth surface is seen at the right upper posterior wall of the gastric fundus. **B:** An approximately 1.7 cm × 1.4 cm, depressed, flat white-red based lesion with oozing hemorrhages is present at the gastric angle.

she was discharged one week later. No evidence of tumor recurrence was found after 14-months of follow-up.

DISCUSSION

GIST concomitant with early gastric cancer has rarely been reported. To the best of our knowledge, a small GIST concomitant with a signet ring cell type of early gastric cancer has never been reported. Although Japanese investigators reported some cases of gastric leiomyoblastoma associated with gastric cancer between 1971 and 2000, CD-117 immunohistochemical staining was not confirmed in those cases. Our 1.1-cm GIST detected by EGD was a small one. The origin of a GIST concomitant with an early gastric adenocarcinoma is unclear. This small GIST showed a positive microscopic finding of *H pylori* microorganisms and a positive CLO test (biopsy urease test) for *H pylori* infections. *H pylori* was implicated as a carcinogen of the stomach by the World Health Organization in 1994. We had previously detected two gastric GIST cases in 2003; both of those had a positive finding of *H pylori* microorganisms and a positive CLO test for *H pylori* infections. However, this finding is more likely an incidental finding rather than a causal association.

GISTs are considered to be a group of mesenchymal neoplasms, and are also the subject of much debate and controversy regarding their nomenclature, histogenesis, criteria for diagnosis, prognostic manifestations, and classification^[1,2]. Studies have revealed that some of these tumors may have tumor markers and features of neural, muscular or vascular endothelial differentiation. The term GIST has been adopted and defined as tumors arising from the stroma with no definite cell line of origin and varying patterns of differentiation^[3]. Our GIST showed a mixed type of smooth muscle-neural origin. Some investigators emphasize the CD117 and CD34 expression in GISTs^[1,4-6]. GISTs are now preferentially defined as tumors with c-kit (CD117) positive mesenchymal spindle cells or epithelioid neoplasms primarily in the gastrointestinal tract, omentum, and mesentery^[4].

Statistically, GISTs are most common in the stomach (60-84.8%), followed by small intestine (10.5-30%), colon and rectum (3.5-5%), and esophagus (1.2-5%)^[1,4]. The most important manifestation of stromal tumors is their

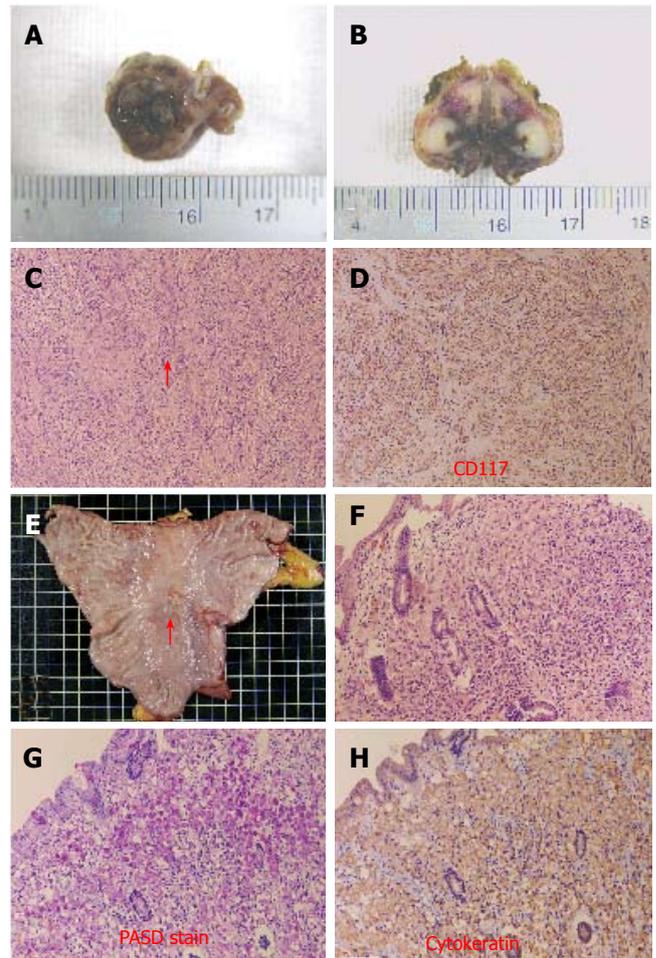


Figure 2 **A** The excised 1.1-cm fundal tumor showing gray fleshy and nodular cut surface; **B** and **C:** Its histologic picture demonstrating whirling bundles of spindle cells with a mitosis (arrow, H&E, ×200); **D:** staining of spindle cells for CD117 (IHC, ×200); **E:** subtotal gastrectomized specimen showing a 1.7 cm × 1.4 cm ulcerative mass at the angularis of the lesser curvature side; **F:** tumor showing signet ring cells within the lamina propria histologically (H&E, ×200); **G:** tumor stained for PAS-diesterase (×200); **H:** tumors stained for cytokeratin (IHC, ×200).

indolent, slow growing nature. The tumors are generally found within the deeper stroma and the submucosa, and are often incidentally found during imaging studies. Our small GIST protruded about half of its mass into the upper posterior wall of the gastric fundus and only a 0.4 cm polypoid mass could be found in the endoscopic field.

GISTs often present with non-specific symptoms, such as nausea, vomiting, abdominal pain, gastrointestinal bleeding, and metastatic diseases. Bleeding is considered as the most common presentation of the clinical course^[2]. Symptoms depend on tumor size and location. In our case, the GIST was totally asymptomatic. Ulcerations seem relevant to the size of GISTs. The size and mitotic score are considered as important diagnostic criteria and prognostic predictive indicators^[1,2]. This GIST was diagnosed as a potential malignancy with a very low risk according to the pathological findings of mitosis (2 per 50 HPF) with combined smooth muscle-neural differentiation and positive stains for CD117 and CD34^[1,2,5-7].

Generally, the preoperative rate of diagnosis of submucosal tumors by an endoscopic biopsy or an imaging

study without skillful examination with an endoscopic ultrasonographic system and angiography is very low. Once a submucosal tumor is suspected, more careful endoscopic procedures, and repeat deep biopsy of the same area to reach the target tumor may have a higher diagnostic rate of histological findings, as our experience attests.

Patients with advanced GISTs which progress rapidly and result in organ destruction have poor prognosis. Therapeutic options for GISTs include surgery and treatment with STI-571 (Gleevec). STI-571 is a small molecule competitive inhibitor of the ATP binding site and demonstrates a high degree of specificity for inhibiting the activity of a small number of related tyrosine kinases: c-Ab1, Bcr-Ab1 (the molecular cause of chronic myeloid leukemia), platelet-derived growth factor receptors^[8-10], and wild-type and mutant c-kit (stem cell factor receptor)^[8,11,13]. This selective activity of STI-571 suggests that it has a relatively narrow target spectrum of anticancer activity. This highly selective molecule has a different therapeutic effect. Most malignant GISTs have mutant c-kit and some studies show that c-kit may be activated by mutation in three domains: extracellular, juxtamembrane and kinase portions (exon 9 or 11 or 13)^[5,6,11,12]. STI-571 has the ability to inhibit signal transduction via c-kit and it is predictable that it should inhibit hematopoietic stem cells, resulting in neutropenia, anemia, thrombocytopenia, GI bleeding or intratumor hemorrhage^[7].

Surgery is still the mainstay treatment for GISTs. Subtotal gastrectomy for the early gastric cancer with local resection for the small GIST was performed simultaneously for our patient with a small GIST concomitant with a IIc type of early gastric cancer.

In conclusion, we have reported a rare case of a small GIST combined with an early gastric cancer. It showed positive microscopic findings of *H pylori* microorganisms, a positive CLO test, and stainings for CD117 and CD34. More GIST cases are required for evaluating the relationship between *H pylori* infections and the etiologies of GIST concomitant with an early gastric cancer. Long-term observation for all GIST cases is greatly needed.

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CASE REPORT

A case of successful management with splenectomy of intractable ascites due to congenital dyserythropoietic anemia type II-induced cirrhosis

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Abstract

The congenital dyserythropoietic anemias comprise a group of rare hereditary disorders of erythropoiesis, characterized by ineffective erythropoiesis as the predominant mechanism of anemia and by characteristic morphological aberrations of the majority of erythroblasts in the bone marrow. Congenital dyserythropoietic anemia type II is the most frequent type. All types of congenital dyserythropoietic anemias distinctly share a high incidence of iron loading. Iron accumulation occurs even in untransfused patients and can result in heart failure and liver cirrhosis. We have reported about a patient who presented with liver cirrhosis and intractable ascites caused by congenital dyserythropoietic anemia type II. Her clinical course was further complicated by the development of autoimmune hemolytic anemia. Splenectomy was eventually performed which achieved complete resolution of ascites, increase of hemoglobin concentration and abrogation of transfusion requirements.

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INTRODUCTION

All types of congenital dyserythropoietic anemias (CDAs) distinctly share a high incidence of iron loading^[1]. As in other forms of anemia with ineffective erythropoiesis, this is due to the upregulation of iron resorption^[2]. Iron accumulation occurs even in untransfused patients and can result in diabetes mellitus, hypogonadotropic hypogonadism and more importantly, heart failure and liver cirrhosis^[3-5].

We have reported about a patient who presented with liver cirrhosis and intractable ascites caused by CDA II. Her clinical course was further complicated by the development of autoimmune hemolytic anemia. Splenectomy was eventually performed, which achieved complete resolution of ascites, increase of hemoglobin concentration and abrogation of transfusion requirements.

CASE REPORT

A 58-year-old female presented to our department because of progressive abdominal distention. She had a history of CDA II that was diagnosed 15 years ago, and received blood transfusion only at the time of diagnosis and never again.

Physical examination revealed mild jaundice and abdominal distention. The edge of the liver extended 3 cm below the right costal margin and marked splenomegaly

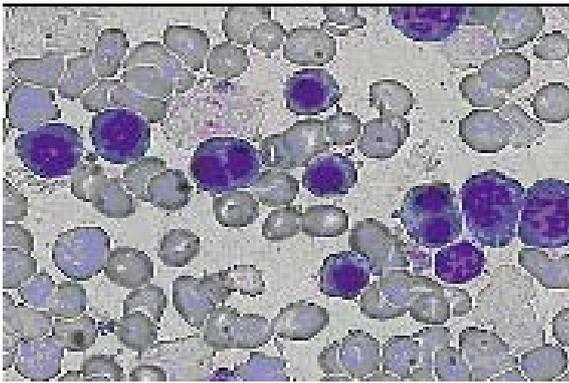


Figure 1 Peripheral blood smear showing binucleated normoblasts.

was also found. Percussion of the abdomen revealed the presence of flank dullness and “shifting”.

The results of laboratory tests were: WBC 7700/ μ L, Hb 9.8 g/dL, MCV 91.7 μ m³, MCH 32.1 pg, PLT 223 000/ μ L, PT 13.9, INR 1.07, PTT 28.0, glucose 120 mg/dL, urea 53 mg/dL, creatinine 0.9 mg/dL, AST 81 U/L, ALT 117 U/L, LDH 176 U/L, protein 7.0 g/dL, albumin 4.3 g/dL, total bilirubin 1.76 mg/dL, unconjugated bilirubin 1.44 mg/dL, ferritin 2 200 ng/mL. Folic acid was 7.4 ng/mL (normal range, >5.38 ng/mL) and vitamin B12 was 352 pg/mL (normal range, 211-911 pg/mL), thus ruling out megaloblastic anemia. Hemoglobin electrophoresis showed normal findings.

Analysis of the ascitic fluid obtained after abdominal paracentesis yielded the following results: absolute polymorphonuclear leukocyte count 100/mm³, albumin 1.0 g/dL (serum-ascites albumin gradient 3.3 g/dL), glucose 128 mg/dL, LDH 43 U/L.

Peripheral blood smear showed anisopoikilocytosis, basophilic stippling and binucleated normoblasts (Figure 1). The Ham's test was negative, possibly because of a small amount of normal sera used. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) of erythrocyte membrane proteins showed that band 3 was narrower and had an accelerated migration rate. Bone marrow examination revealed marked erythroid hyperplasia, while approximately 30% of the mature erythroblasts were binucleate.

A liver biopsy documented cirrhosis with marked siderosis of hepatocytes as well as Kupffer cells and portal macrophages (Figure 2). Genetic testing for mutations of the HFE gene revealed heterozygosity for the H63D mutation, whereas the patient was negative for the C282Y mutation. Other causes of cirrhosis were thoroughly excluded, based on the medical history of the patient, negative virology for hepatitis B and C, normal immunologic tests (including testing for the presence of antinuclear antibodies, anti-smooth muscle cell antibodies and anti-mitochondrial antibodies) and liver biopsy findings.

Upper endoscopy revealed the presence of esophageal varices. An abdominal ultrasonographic study showed cholelithiasis. A cardiac ultrasonographic examination showed concentric hypertrophy and impaired diastolic function of the left ventricle, left atrium dilatation, mitral

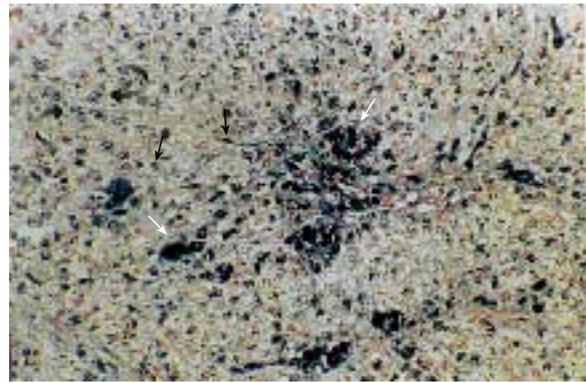


Figure 2 Liver biopsy showing marked siderosis of hepatocytes (curved arrows), Kupffer cells (arrowheads with ball) and portal macrophages (arrows) (Perl's stain, \times 200).

valve regurgitation and right ventricle dilatation with tricuspid valve regurgitation and pulmonary hypertension (right ventricular systolic pressure: 5332 Pa). Magnetic resonance imaging was performed in order to determine heart and liver iron concentration using a unit (Magnetom Impact; Siemens, Germany) operating at a field strength of 1.0 Tesla. We used an electrocardiogram-triggered gradient-recalled-echo sequence for the evaluation of both myocardial and liver iron. Iron content measurement was based on a modification of the method of Bonkovsky, calculating the natural logarithm of the ratio of the mean signal intensity of the liver or heart respectively by that of the standard deviation of the mean of the signal intensity of the overlying air [\ln signal intensity ratio, \ln (SIR); normal values for both heart and liver, > 3.2]^[6]. The \ln (SIR) values obtained for the heart and liver were 2.66 and 1.26 respectively.

Since the patient persistently refused to undergo iron chelation therapy with desferrioxamine, deferiprone (75 mg/kg per day in three divided doses) was initiated, spironolactone (200 mg once daily) and furosemide (80 mg once daily) were also prescribed but dose escalation was not possible because of deterioration in renal function. Since the patient also did not decrease her salt intake, large volume of paracentesis (approximately 8-10 L of ascitic fluid) had to be performed approximately every 10 d due to the development of refractory ascites.

Soon after her presentation, the patient began to require frequent blood transfusions. Direct antiglobulin test was positive due to the presence of IgG autoantibodies. Extensive investigation, including repeated immunologic tests to rule out autoimmune diseases and imaging studies to rule out occult malignancy, excluded secondary causes of autoimmune hemolytic anemia (AIHA). The patient was treated with prednisone and subsequently with intravenous immunoglobulin, both failed to produce a significant response. Therefore, we performed splenectomy in order to control both CDA and AIHA as well as proximal splenorenal shunt in order to relieve portal hypertension and ascites. Surgery and recovery were uneventful, but a Doppler examination performed immediately after the operation showed complete shunt occlusion.

During the 10-mo post-operative follow-up period, the

patient was transfusion-independent and ascites did not relapse although diuretics were discontinued. Hemoglobin concentration was persistently above 12.0 g/dL and unconjugated bilirubin was normalized. Nevertheless, the concentration of ferritin remained elevated at 1 800 ng/mL. A new cardiac ultrasonographic examination disclosed regression of the right ventricle dilatation and no signs of pulmonary hypertension (right ventricular systolic pressure: 1 999.5 Pa). Follow-up magnetic resonance imaging of the heart and liver showed heart siderosis within normal limits but no change in liver siderosis with her ln (SIR) values being 4.04 and 1.38, respectively.

DISCUSSION

The evolution of CDA II is doomed by iron overloading, which is also seen in patients with no need for transfusion and is mainly due to increased iron resorption. Lethal organ damage of the liver and myocardium is observed in untreated patients^[7]. The rate of iron accumulation seems not to be affected by *HFE* gene polymorphism^[8,9]. Our patient was heterozygous for the H63D mutation, which may contribute to increased hepatic iron levels but does not result in iron overload in the absence of the main mutation responsible for hereditary hemosiderosis (C282Y)^[10-12].

Hemosiderosis in CDA type II is treated with desferrioxamine which is usually introduced as soon as ferritin reaches the concentration of 1 000 ng/mL^[13,14]. Normal ferritin concentrations were reached in all the patients with satisfactory compliance^[4] a full-stop. Nevertheless, desferrioxamine must be given 5 to 7 d per week by a prolonged subcutaneous infusion^[15]. On the other hand, deferiprone is an alternative chelator that is orally active and more effective than desferrioxamine in the removal of myocardial iron^[16]. Indeed, heart siderosis returned to normal levels in our patient, even though the reduction in ferritin levels and liver iron overload was not so pronounced.

In CDA II, splenectomy leads to a moderate and sustained increase in hemoglobin concentration and decrease of hemolysis^[4,8]. Though splenectomy has these benefits, recommendation for the operation is not uniform among the hematologists^[8]. Furthermore, splenectomy does not prevent further iron loading, even in those patients with their hemoglobin concentrations becoming nearly normal as observed in our patient^[4].

The concurrence of CDA II and AIHA has been described only once, thus suggesting chance occurrence^[17]. In AIHA, splenectomy has the distinct advantage over other therapeutic options in that it has the potential for complete and long-term remission. Available data suggest that it triggers remission in more than 50% of patients^[18]. Excellent responses or improvements are maintained during a mean follow-up period of 33 or 73 mo, respectively^[19]. Accordingly, splenectomy should be considered in patients who do not respond adequately to corticosteroids^[20].

In patients with refractory ascites, both transjugular intrahepatic portosystemic shunt (TIPS) and surgical portal systemic shunt have been used^[21]. TIPS is a widely accepted

percutaneous interventional procedure for treating complications of portal hypertension. An experienced skillful team, however, is necessary to ensure the technical success of TIPS and to avoid its potential procedural complications^[22]. Shunt dysfunction is also a major problem, since over 50% of TIPS develop stenosis within 1 year and therefore, close shunt surveillance and frequent re-treatment are required with ensuing high costs^[23-25]. Furthermore, hepatic encephalopathy is another important complication of cirrhotic patients with refractory ascites treated with TIPS, more than 40% of these patients develop encephalopathy^[26,27]. Nevertheless, calibrated prosthesis can partly prevent TIPS complications^[28].

The role of surgery in the treatment of portal hypertension remains a complex and highly controversial issue. Several factors must be considered when surgical options are to be entertained, including origin and extent of liver disease, response to prior medical treatment, and possibility of future liver transplantation^[29]. Splenectomy was mandatory in our patient in order to control CDA and particularly AIHA. However, splenectomy alone is inappropriate for the treatment of portal hypertension and does not relieve ascites^[30,31]. In contrast, portosystemic shunting is efficient in clearing ascites, but is associated with a high rate of encephalopathy and liver failure^[29,32,33]. The indications for portosystemic shunting are therefore limited for the treatment of intractable ascites and portosystemic shunting should be performed only in patients with good liver function or when all other treatments fail^[29,32]. Since our patient had preserved hepatic synthetic capacity, we chose to combine splenectomy with portosystemic shunting in order to relieve ascites and thus performed proximal splenorenal shunt. Compared to selective shunts, the central or proximal splenorenal shunt does not differ in operative mortality rates, 5-year survival rates, development of individual episodes of post-operative encephalopathy, or chronic post-operative encephalopathy^[34]. Unfortunately, shunt occlusion may occur immediately after the operation. It is thus difficult to explain the complete remission of ascites and we can only postulate that a reduction in the blood flow into the portal system from the greatly enlarged spleen may result in a decrease in portal pressure. This reduction in portal pressure may also contribute to the amelioration of pulmonary hypertension as observed in our patient.

In conclusion, even in transfusion-independent patients with CDA II, iron overload can result in dramatic manifestations, posing major therapeutic difficulties. Therefore, ferritin levels should be controlled in all the patients with CDA II, because iron overload may approach risk levels at any age^[1]. Iron depletion has to be started at the latest when ferritin approaches a level of 1 000 ng/mL to avoid the devastating complications of excessive iron accumulation^[4].

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CASE REPORT

Five-year survival following a medial pancreatectomy for an invasive ductal carcinoma from the body of the pancreas

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Abstract

We report a rare case of a patient who survived for 5 years after undergoing a medial pancreatectomy for invasive ductal carcinoma originating from the body of the pancreas. A 63-year-old woman was diagnosed as a small cancer of the pancreatic body, and surgery was performed. Even though the tumor was a carcinoma, its small size prompted us to perform a medial pancreatectomy with regional lymph nodes dissection. Additional chemoradiation was performed and, five years after surgery, the patient is well with no signs of recurrence. Medial pancreatectomy for invasive ductal carcinoma has not ever been reported. Furthermore, long-term survival after a lumpectomy for invasive ductal carcinoma has never been reported in the literatures. The current case suggests that long-term survival in patients with invasive ductal carcinoma of the pancreas may be associated with the pathological or biological features of pancreatic carcinoma.

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Key words: Ductal carcinoma; Medial pancreatectomy; Pancreatic carcinoma

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INTRODUCTION

Invasive ductal carcinomas that originate from the body

or tail of the pancreas are often unresectable because of unfortunate delays in tumor diagnosis. Most institutions continue to report resection rates for standard surgical procedures and 3-year or 5-year survival rates of no more than 20%^[1]. Medial pancreatectomy is a segmental pancreatectomy for the treatment of benign lesions in the neck and body of the pancreas^[2]. We report the extremely rare case of a patient who survived for 5 years after undergoing a medial pancreatectomy for invasive ductal carcinoma originating from the body of the pancreas.

CASE REPORT

A 63-year-old woman was admitted to our hospital for an evaluation of a dilatation in her main pancreatic duct. She had been diagnosed as having diabetes mellitus at the age of 53 years. Blood sugar control prompted her previous doctor to perform an abdominal computed tomography examination, which showed an apparent dilatation of the main pancreatic duct in the body and tail of the pancreas. Neither contrast-enhanced computed tomography, magnetic resonance imaging nor endoscopic ultrasonography revealed the cause of the dilatation, however, magnetic resonance cholangiopancreatography showed that the main pancreatic duct with beaded dilatation suddenly became occluded in the body of the pancreas. A clinical diagnosis of non-detectable, small cancer of the pancreatic body was made, and surgery was performed. An intraoperative ultrasonography examination depicted a round hypoechoic mass, measuring 1.0 cm in diameter, in the body of the pancreas. No infiltration to the vessels or metastatic lesions was detected. Even though the tumor was a carcinoma, its small size prompted us not to perform a Whipple procedure or a left pancreatectomy. Instead, a medial pancreatectomy, 4 cm along the long pancreatic axis, and an independent dissection of the lymph nodes around the common hepatic artery and proximal splenic artery were performed. The tail of the pancreas was anastomosed using a Roux loop. The tumor was histologically diagnosed as a well-differentiated adenocarcinoma, with a maximum diameter of 1.0 cm (Figure 1); one peripancreatic lymph node containing a metastasis was observed (Figure 2). When examined under low magnification, lymphocyte aggregation around the cancer stroma was observed, immunohistochemically, the tissue stained positive for both CD4 and CD8. The patient's postoperative course was uneventful; adjuvant chemoradiation was performed for 4 wk. The external-beam radiation doses were delivered using conventional

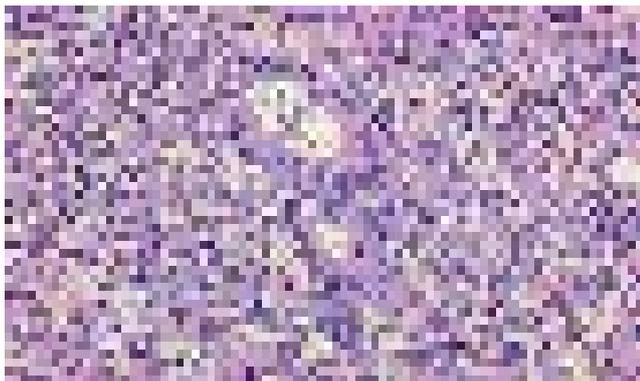


Figure 1 Under high magnification, the tumor is histologically diagnosed as a well-differentiated adenocarcinoma and is surrounded by lymphocyte aggregation.

fractionation: 2 Gy, 5 fractions a week. Concomitant chemotherapy was also performed on the first and fifth days of every week (10mg CDDP, 1KE OK432). In addition, she received a 120-h continuous infusion of 625 mg of 5-fluorouracil per week during the radiotherapy. The patient is well with no signs of recurrence five years after surgery.

DISCUSSION

Many surgeons perform extended radical surgeries, including an extended lymphadenectomy and retroperitoneal soft-tissue clearance, for various stages of pancreatic cancer; however, a survival benefit has not been demonstrated^[3]. Even after a curative Whipple resection is performed, adjuvant treatments like radiotherapy and 5-FU treatment are often performed in the USA. So, Jeekel stated that a Whipple procedure was inadequate for pancreatic head cancer and regarded this procedure to be a lumpectomy rather than a curative procedure^[4]. A medial pancreatectomy for invasive ductal carcinoma should also be regarded as a lumpectomy.

The main problem associated with performing a medial pancreatectomy is the need for careful patient selection^[2]. In the present case, a very small tumor was visualized in the neck or right margin of the body of the pancreas. Preoperatively, evidence suggesting an invasive ductal carcinoma was minimal. The involvement of the main pancreatic duct enabled us to avoid enucleation. In conventional pancreatectomies, a pancreaticoduodenectomy or a near-total left pancreatectomy may be required. To avoid postoperative or late complications from a major pancreatic resection, we performed a medial pancreatectomy and independent lymph-node dissection in the present case. Medial pancreatectomy for invasive ductal carcinoma has not ever been reported. Furthermore, long-term survival after a lumpectomy for invasive ductal carcinoma has never been reported in the literatures, to the best of our knowledge.

Large multivariate analyses of resected pancreatic adenocarcinoma have proven that lymph node metastasis serve as prognostic features, however, Fortner *et al*^[5] reported that the presence of lymph node metastasis did not significantly influence survival after regional pancreatectomies for various stages of pancreatic cancer. In tumor

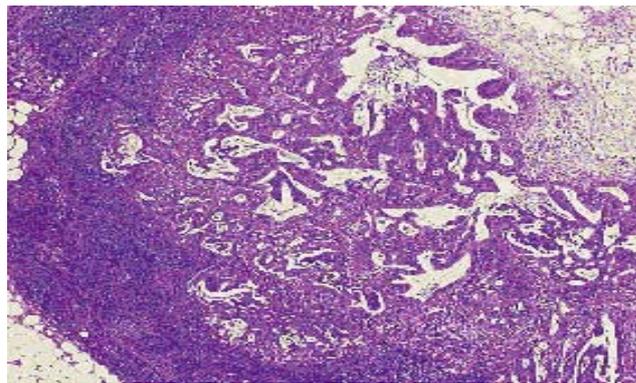


Figure 2 Cancer metastasis in a lymph node close to the pancreatic body.

biology, the existence of carcinoma cells in a lymph node is regarded as indicating metastatic invasion through the lymphatic ducts out of primary organ. In the present case, the peripancreatic lymph-node metastasis did not preclude long-term survival. Thus, long-term survival may not be precluded by the presence of lymph node metastasis, otherwise, extended radical surgery can not contribute to the treatment of metastatic invasion through the lymphatic ducts. The prognosis of patients with invasive ductal carcinoma of the pancreas who have survived for 5 years may be the same, regardless of whether an extended radical surgery or a lumpectomy is performed.

Many patients with even stage I diseases have recurrences after resection. Fortner *et al*^[5] reported in 1996 that the 5-year survival rate after a regional pancreatectomy was 33% in 12 patients with ductal adenocarcinoma of the pancreatic head, in whom the tumor measured 2.5 cm or less in diameter. Some patients with advanced diseases, however, have survived longer than 5 years after their initial treatment. The pathological and biological features of the 5-year survivors varied.^[6] In the present case, peritumoral lymphocyte aggregation was observed, and CD4+ and CD8+ T cells were noted using immunohistochemistry. Fukunaga *et al*^[7] reported that the presence of CD4+ and CD8+ T cells in pancreatic adenocarcinoma serves as a good indicator of a favorable outcome after surgical treatment.

In summary, we report a patient with invasive ductal carcinoma from the body of the pancreas that survived for more than five years after undergoing a medial pancreatectomy. The current case suggests that complete surgical extirpation of early-stage carcinomas of the pancreas accompanied with adjuvant therapy can be long-term survival may be associated with the pathological or biological features of pancreatic carcinoma.

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Adult case of an omphalomesenteric cyst resected by laparoscopic-assisted surgery

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Abstract

This report describes an extremely rare adult case of an omphalomesenteric cyst resected by laparoscopic-assisted surgery. A 29-years-old Japanese man was referred and admitted to Kyushu University Hospital because of an abdominal mass and an elevated serum CEA (carcinoembryonic antigen) level (21.3 ng/mL) in August 2001. Abdominal CT and US demonstrated a cystic mass with septum and calcification. Laparoscopy showed a large mass to be attached to his abdominal wall, measuring 110 mm x 70 mm x 50 mm and filled with mucus. The mass was resected by laparoscopic-assisted surgery. The histological findings of its wall showed fibromuscular tissue, adipose tissue, calcification, and an intestinal structure. It was finally diagnosed to be an omphalomesenteric cyst.

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Key words: Omphalomesenteric cyst; Laparoscopic-assisted surgery

Sawada F, Yoshimura R, Ito K, Nakamura K, Nawata H, Mizumoto K, Shimizu S, Inoue T, Yao T, Tsuneyoshi M, Kondo A, Harada N. Adult case of an omphalomesenteric cyst resected by laparoscopic-assisted surgery. *World J Gastroenterol* 2006; 12(5): 825-827

INTRODUCTION

The omphalomesenteric duct remnant is one of the rare congenital anomalies associated with the primitive yolk stalk. Most omphalomesenteric duct remnants tend to be Meckel's diverticulum, however, the occurrence of an omphalomesenteric cyst is very rare. An omphalomesenteric duct remnant induces several symptoms, such as intestinal obstruction, abdominal pain, melena, and umbilical hernia. These symptoms tend to occur most frequently during the childhood years. In this report, we have described an extremely rare adult case of omphalomesenteric cyst resected by laparoscopic-assisted surgery.

CASE REPORT

A 29-year-old male patient visited Aso Iizuka Hospital with the complaint of an abdominal mass of one month duration in July 2001. The laboratory data revealed an elevated CEA level (21.3 ng/mL). Abdominal CT showed a cystic mass, suspected of being a mesenteric cyst. In August 2001, he was referred and admitted to Kyushu University Hospital for further management. Physical examinations showed no abnormalities except for a fist-sized, elastic soft mass in his umbilical region. The laboratory data demonstrated elevations in the ALT (32I U/L), CEA (7.9 ng/mL, normal range <3.2 ng/mL), and CA19-9 (62.6 U/mL, normal range <37.0 U/mL) levels. However, the serum CA125 and alpha fetoprotein (AFP) levels were both within the normal limits. Abdominal US and CT showed a cystic mass with septum and calcification, measuring 100 mm x 80 mm x 50 mm in size (Figure 1). MRI (T2-WI) showed a cystic mass with septum, which was suspected to be a mesenteric cyst (Figure 2). A barium small-bowel enema showed no abnormality except for extraduodenal oppression. Angiography showed an avascular area in the mass. Our preoperative diagnosis was mesenteric cyst.

Laparoscopic-assisted surgery was performed for the purpose of making a precise diagnosis and resection, because the possibility of a CEA-producing malignant tumor first had to be ruled out. A laparoscope was inserted through a 1 cm-trocar site at the median upper abdomen. Laparoscopy showed the mass to be attached to his abdominal wall at the median hypogastrium (Figure 3), and two ligaments, considered to be remnants of the umbilical arteries, at its attached portion. The mass had no communication with his small bowel. There was no evidence

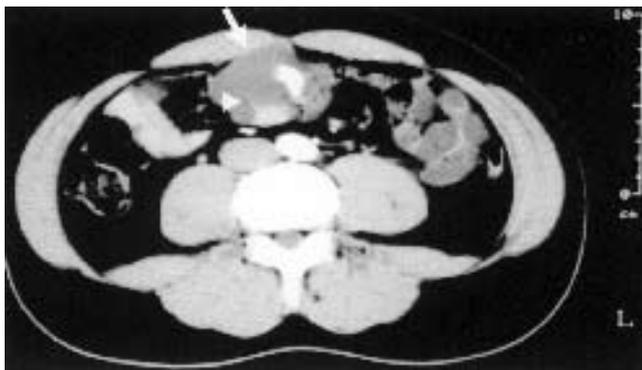


Figure 1 Abdominal CT demonstrating a cystic mass (arrow), measuring 100 mm x 80 mm x 50 mm in size, with septum (arrow head) and calcification in the umbilical region.



Figure 2 MRI (T2-WI) showing a cystic mass with septum.



Figure 3 Laparoscopy showing the mass to be attached to abdominal wall.

of a persistent urachus. Next, an additional two ports were made with one at the right upper abdomen and another at the right lateral abdomen. The mass was then resected from his abdominal wall using laparoscopic scissors and electrocautery. The mass was removed through a 5-cm median incision made at his lower abdomen. The mass measured 110 mm x 70 mm x 50 mm in size (Figure 4), and weighed 160 g. This cystic mass was filled with mucus (Figure 5). The histological findings demonstrated the cystic wall to be composed of fibromuscular tissue, adipose tissue, focal calcification and an intestinal type mucosa with goblet cells, accompanied by ulceration with a regenerative



Figure 4 Mass measuring 110 mm x 70 mm x 50 mm in size.



Figure 5 Cystic mass filled with mucus.

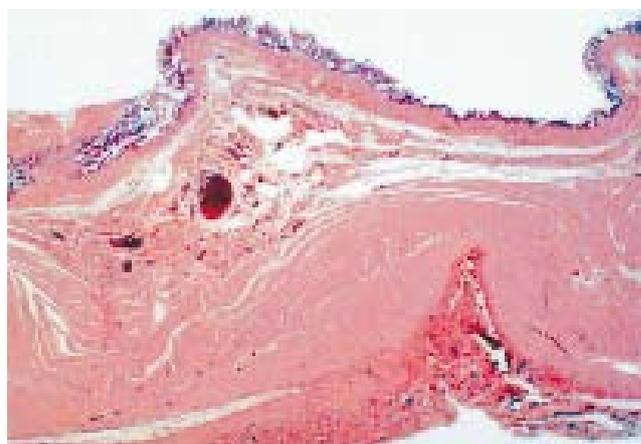


Figure 6 Histological features showing cystic wall to be composed of fibromuscular tissue, adipose tissue, focal calcification, and an intestinal structure, lined by an intestinal type mucosa with goblet cells, accompanied by ulceration with a regenerative epithelium.

epithelium (Figure 6). The histological findings showed no evidence of malignancy. It was finally diagnosed to be an omphalomesenteric cyst. Six months after the operation, the serum CEA and CA19-9 levels both became normal (0.7 ng/mL and 12.46 U/mL, respectively).

DISCUSSION

Omphalomesenteric duct remnants (vitelline duct anomalies) have been reported to be congenital anomalies associated with the primitive yolk stalk. The omphalomesenteric duct is the embryonic structure connecting the primary yolk sac to the embryonic midgut that normally closes off spontaneously at about 5-9 wk of gestation^[1]. The failure of such closure may result in various lesions occurring in the omphalomesenteric duct remnants, such as Meckel's diverticulum, complete umbilical enteric fistula, incomplete umbilical sinus, omphalomesenteric cyst, or umbilical mucosal polyp^[2]. Most of omphalomesenteric duct remnants (67%) tend to develop into Meckel's diverticulum, however,

omphalomesenteric cyst is also rarely observed^[3]. Vane *et al*^[1] reported only three cases of omphalomesenteric cyst in 217 children with omphalomesenteric duct remnants. The etiology of omphalomesenteric cyst is a persistent lesion of the intermediate duct with closure at both ends, leaving a cyst which can attach itself to the umbilicus, bowel, or both^[4].

Omphalomesenteric duct remnants may persist in approximately 2% of infants^[4,5]. Vane *et al*^[1] reported the male-female ratio in a 217 remnant series to be 2:1. Yamada *et al*^[6] reported the male-female ratio to be 2.8:1 in a Japanese series of 65 cases. The male-female ratio of omphalomesenteric cyst was reported to be 4:1 which correlates with the previously reported male predominance in other omphalomesenteric duct anomalies^[7].

Common symptoms of omphalomesenteric duct remnants include abdominal pain, rectal bleeding, intestinal obstruction, umbilical drainage, and umbilical hernia^[3], and all these symptoms appear to be age-dependent. In addition, most of the symptoms usually appear before the age of 4 years^[1]. Eighty-five percent of infants younger than 1 month and 77% of children aged 1 month to 2 years had a symptomatic presentation^[1]. It was also reported that 40% of the children with this anomaly had symptomatic lesions, while this anomaly is usually asymptomatic in adults^[1]. Adult cases of omphalomesenteric cyst are extremely rare. A surgical resection is necessary for symptomatic omphalomesenteric duct remnants, but not necessary for asymptomatic omphalomesenteric duct remnants^[8,9]. In our case, although asymptomatic omphalomesenteric cyst was incidentally found, it was removed because the possibility of a CEA-producing malignant tumor and small-bowel

obstruction had to be ruled out^[1]. The elevated tumor markers (CEA, CA19-9) all returned to normal levels after the resection. However, its histological findings showed no evidence of malignancy. The etiology of such elevated tumor markers remains to be elucidated.

In our case, the omphalomesenteric cyst was resected safely by laparoscopic-assisted surgery. Recent advances in diagnostic modalities have resulted in an increased incidental discovery of asymptomatic congenital anomalies. In such cases, the use of laparoscopic surgery is considered to be an effective, safe and less invasive treatment.

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LETTERS TO THE EDITOR

Clinical practice guidelines for hepatocellular carcinoma: the first evidence based guidelines from Japan

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TO THE EDITOR

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer death in Japan, ranked 3rd in males and 5th in females. Thanks to recent progress, there are several definitive treatment modalities available for HCC, including surgery (liver resection and transplantation), ablation therapy, and transarterial chemoembolization (TACE). It is fortunate for both patients and doctors to have multiple treatment options, however, there have been very few

evidence-based guidelines for decision-making^[1]. Supported by the Japanese Ministry of Health, Labour and Welfare, we have compiled the “Clinical practice guidelines for hepatocellular carcinoma”^[2]. This set of guidelines covers 6 fields for HCC, including prevention, diagnosis and surveillance, surgery, chemotherapy, TACE, and ablation therapy. We have surveyed 7192 publications on HCC extracted mainly from MEDLINE (1966-2002), and selected 334 articles to form 58 pairs of research questions and recommendations. For convenience in practical use, we have also created algorithms for the surveillance and treatment of HCC.

The algorithm for the treatment of HCC was based on the evidence from the selected articles above and modified according to the current status in Japan, where liver resection for HCC is safe with less than 1% mortality, and cadaveric donors for liver transplantation are extremely difficult to obtain (Figure 1). This algorithm was devised on the basis of three factors, namely, degree of liver damage^[3], number of tumors, and diameter of the tumors. In patients with the severity of the liver damage categorized into class A or B: (1) If there is only one tumor, liver resection is recommended, irrespective of the diameter of the tumor. Ablation therapy may also be selected if the severity of liver damage is class B and the diameter of the tumor is not more than 2 cm; (2) From 2 to 3 tumors of no more than 3 cm in diameter, liver resection or ablation therapy is recommended; (3) From 2 to 3 tumors with a diameter of 3 cm or more, liver resection or TACE is recommended; (4) For 4 or more tumors, TACE or he-

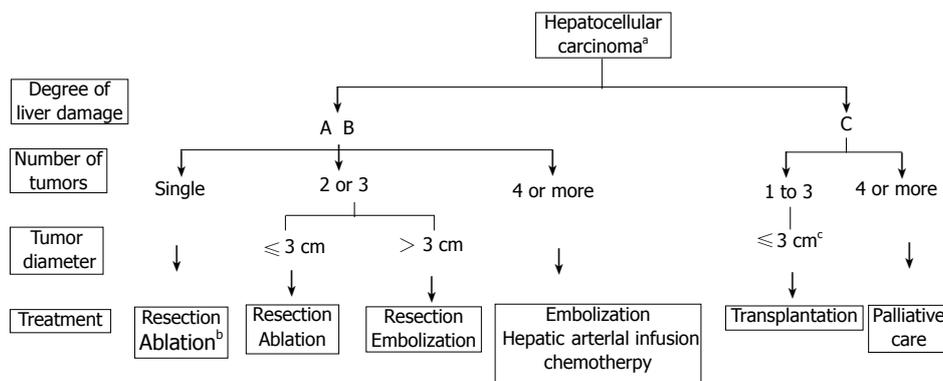


Figure 1 Treatment algorithm for hepatocellular carcinoma (cited from Ref.2 with permission). ^aPresence of vascular invasion or extrahepatic metastasis to be indicated separately. ^bSelected when the severity of liver damage is class B and tumor diameter is no greater than 2 cm. ^cTumor diameter should be no greater than 5 cm when there is only one tumor.

patric arterial infusion chemotherapy is recommended. For patients with class C liver damage: (1) If the tumor condition is within the so-called Milan criteria^[4], liver transplantation is recommended; (2) If the number of tumors is 4 or more, palliative treatment is recommended. For patients with class A liver damage accompanied by vascular invasion, liver resection may be selected, and for patients with extra-hepatic metastasis, chemotherapy may be selected.

English translation for this guideline will be released on the website of The Japan Society of Hepatology (<http://www.jsh.or.jp/>) soon.

Co-investigators for “Clinical practice guidelines for hepatocellular carcinoma” (in alphabetical order): Shigeki Arii, MD, Shunji Futagawa, MD, Yuji Itai, MD, Shuichi Kaneko, MD, Seiji Kawasaki, MD, Ken-ichi Kobayashi, MD, Hiroshi Matsuyama, PhD, Masatoshi Okazaki, MD, Kiwamu Okita, MD, Masao Omata, MD, Yukihisa Saida,

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LETTERS TO THE EDITOR

VEGF in hepatocellular carcinoma and surrounding cirrhotic liver tissues

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TO THE EDITOR

We read with a great interest the recent work of Deli *and colleagues*.^[1] in the *World Journal of Gastroenterology* reporting vascular endothelial growth factor (VEGF) expression in hepatocellular carcinoma (HCC) and cirrhotic liver tissues. This well-documented work shows that VEGF was significantly higher in surrounding cirrhotic liver tissues than in HCC. Authors assessed VEGF expression using immunohistochemistry. The immunohistochemical staining is an efficient tool to assess the percentage of cells stained positively for VEGF but is not really efficient to estimate their true VEGF content. Evaluation of the VEGF protein by an enzyme-linked immunosorbent assay (ELISA) has been reported, by us and others^[2-5], to be an efficient tool in order to assess tissue VEGF expression. We have, thus, tested whether the ELISA method might be an efficient tool in order to confirm data reporting higher amounts of VEGF in surrounding cirrhotic liver tissues than in HCC. Deli *and colleagues*.^[1] also correctly pointed out that basic fibroblast growth factor (bFGF) has been reported to act cooperatively on VEGF expression^[6]. We have, thus, also assessed bFGF tissue levels in order to

search for a putative link between VEGF and bFGF levels in cirrhotic tissues.

The procedure of the present study followed the rules edited by the French National Ethics. Between January 1999 and December 2004, 12 consecutive (10 males and 2 females, mean age 67 ± 4 years, range 36-83 years) patients with an HCC developed in a cirrhosis underwent resection in the Department of Surgery of Limoges' CHU. Specimens for the HCC tissue and the surrounding cirrhotic liver tissue were obtained during the surgical procedure and frozen at -80°C until used. Cirrhosis was present in all cases with the cause being alcoholism or hepatitis C. Histologically, there was 5 well-differentiated HCC, 4 moderately differentiated HCC, and 3 poorly differentiated HCC. Tissue samples were homogenized in potassium phosphate buffer and VEGF and bFGF dosages were performed by specific ELISA assays (DuoSet®, R&D Systems Europe) according to the manufacturer's recommendations. Results in pg per mg of wet weight are reported as means \pm SEM of 12 patients. Differences in mean content of VEGF and bFGF among tumour and the surrounding cirrhotic tissue were analysed by the Student's *t*-test for paired data. A $P < 0.05$ was considered statistically significant. Correlations between bFGF and VEGF values were assessed using linear regression analysis. A $P < 0.05$ and $r > 0.5$ were considered statistically significant.

As shown in Figure 1A, VEGF contents were significantly elevated ($P = 0.01$) in the surrounding cirrhotic liver tissues (18.9 ± 2.1 pg/mg, range 2-33 pg/mg) than in HCC tissues (13.6 ± 1.9 , range 2-25 pg/mg). As shown in Figure 1B, bFGF contents were significantly elevated ($P = 0.02$) in the surrounding cirrhotic liver tissues (55.4 ± 6.0 pg/mg, range 15-94 pg/mg) than in HCC tissues (35.2 ± 8.5 , range 4-63 pg/mg). While bFGF and VEGF levels were not correlated in HCC ($r = 0.02$, $P = 0.66$), bFGF and VEGF were strongly correlated in cirrhotic liver tissues ($r = 0.85$, $P = 0.0005$).

VEGF and bFGF amounts are elevated in cirrhotic liver tissue as compared with HCC ones. VEGF results obtained with the ELISA method corroborate those of Deli *and colleagues*.^[1] concerning VEGF expression using immunohistochemistry. Immunohistochemistry and ELISA are, thus, two complementary methods that can be used in concert and bring concordant results. However, in our opinion, the main advantage of the ELISA method is the possibility to test several factors into the same tissue extract in order to highlight potential links between factors such as those found between VEGF and bFGF in the cirrhotic tissues (but not in HCC ones). As suggested by Deli

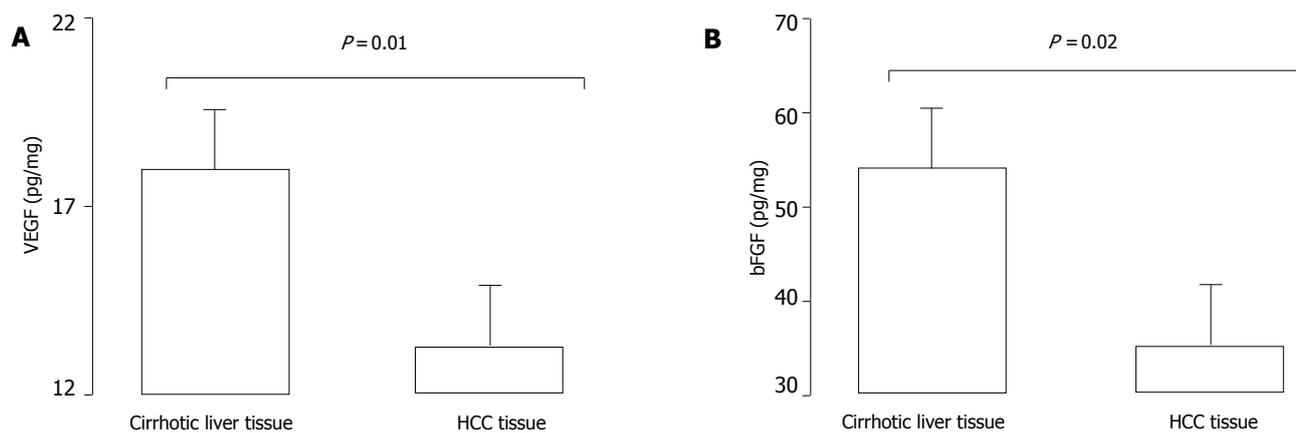


Figure 1 VEGF and bFGF in HCC and surrounding cirrhotic liver tissues. Specimens of HCC and surrounding cirrhotic tissues were obtained during the surgical procedure. VEGF (**A**) and bFGF (**B**) amounts were determined by ELISA. Results are reported as means±SEM of 12 patients. Statistical analysis was performed using the Student's *t* test for paired samples.

and colleagues, VEGF (but also bFGF) may play an important role in the angiogenesis of liver cirrhosis. The higher expression of VEGF and bFGF suggested a possible role of angiogenesis in HCC carcinogenesis. Anti-angiogenic therapy potentially targeting VEGF and bFGF pathways could promise new strategies for the treatment of HCC.

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Meetings

MAJOR MEETINGS COMING UP

Digestive Disease Week
107th Annual Meeting of AGA, The American
Gastroenterology Association
May 20-25, 2006
Loas Angeles Convernition Center, California
www.ddw.org

EVENTS AND MEETINGS IN THE UPCOMING 6 MONTHS

10 th World Congress of the International Society
for Diseases of the Esophagus (ISDE 2006)
February 22-25, 2006
Adelaide
isde@sapmea.asn.au
www.isde.net

EASL 2006 - The 41st Annual Meeting
April 26-30, 2006
Vienna, Austria

International Gastrointestinal Fellows Initiative
February 22-24, 2006
Banff, Alberta
CAGOffice@cag-acg.org
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Canadian Digestive Disease Week
February 24-27, 2006
Banff, Alberta
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European Multidisciplinary Colorectal Cancer
Congress 2006
February 12-14, 2006
Berlin
info@congresscare.com
www.colorectal2006.org

ILTS 12th Annual International Congress
May 3-6, 2006
Milan
www.ilsts.org

World Congress on Gastrointestinal Cancer
June 14-17, 2006
Barcelona, Spain
c.chase@imedex.com

5th International Congress of The African Middle
East Association of Gastroenterology
February 24-26, 2006
Sharjah
infoevent@infomedweb.com
www.infomedweb.com

Digestive Disease Week 2006
May 20-25, 2006
Los Angeles
www.ddw.org

Annual Postgraduate Course
May 25-26, 2006
Los Angeles, CA
www.asge.org/education

EVENTS AND MEETINGS IN 2006

10th World Congress of the International Society
for Diseases of the Esophagus (ISDE 2006)
February 22-25, 2006
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isde@sapmea.asn.au
www.isde.net

10th International Congress of Obesity
September 3-8, 2006
Sydney
enquiries@ico2006.com
www.ico2006.com

EASL 2006 - The 41st Annual Meeting
April 26-30, 2006
Vienna, Austria

International Gastrointestinal Fellows Initiative
February 22-24, 2006
Banff, Alberta
CAGOffice@cag-acg.org
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Canadian Digestive Disease Week
February 24-27, 2006
Banff, Alberta
CAGOffice@cag-acg.org
www.cag-acg.org

Prague Hepatology Meeting 2006
September 14-16, 2006
Prague
veronika.revicka@congressprague.cz
www.czech-hepatology.cz/phm2006

European Multidisciplinary Colorectal Cancer
Congress 2006
February 12-14, 2006
Berlin
info@congresscare.com
www.colorectal2006.org

World Congress on Controversies in Obesity,
Diabetes and Hypertension (CODHy)
October 25-28, 2006
Berlin
codhy@codhy.com
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ILTS 12th Annual International Congress
May 3-6, 2006
Milan
www.ilsts.org

XXX pan-american congress of digestive diseases
November 25-December 1, 2006
Cancun
amg@gastro.org.mx
www.gastro.org.mx

World Congress on Gastrointestinal Cancer
June 14-17, 2006
Barcelona, Spain
c.chase@imedex.com

5th International Congress of the African Middle
East Association of Gastroenterology
February 24-26, 2006
Sharjah
infoevent@infomedweb.com
www.infomedweb.com

7th World Congress of the International Hepato-
Pancreato-Biliary Association
September 3-7, 2006
Edinburgh
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www.edinburgh.org/conference

Digestive Disease Week 2006
May 20-25, 2006
Los Angeles
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October 27-31, 2006
Boston, MA

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|--------|--|--|--|
| 1 | 4 days | 4 d | In figures, tables and numerical narration |
| 2 | 4 days | four days | In text narration |
| 3 | day | d | After Arabic numerals |
| 4 | Four d | Four days | At the beginning of a sentence |
| 5 | 2 hours | 2 h | After Arabic numerals |
| 6 | 2 hs | 2 h | After Arabic numerals |
| 7 | hr, hrs, | h | After Arabic numerals |
| 8 | 10 seconds | 10 s | After Arabic numerals |
| 9 | 10 year | 10 years | In text narration |
| 10 | Ten yr | Ten years | At the beginning of a sentence |
| 11 | 0,1,2 years | 0,1,2 yr | In figures and tables |
| 12 | 0,1,2 year | 0,1,2 yr | In figures and tables |
| 13 | 4 weeks | 4 wk | |
| 14 | Four wk | Four weeks | At the beginning of a sentence |
| 15 | 2 months | 2 mo | In figures and tables |
| 16 | Two mo | Two months | At the beginning of a sentence |
| 17 | 10 minutes | 10 min | |
| 18 | Ten min | Ten minutes | At the beginning of a sentence |
| 19 | 50% (V/V) | 500 mL/L | |
| 20 | 50% (m/V) | 500 g/L | |
| 21 | 1 M | 1 mol/L | |
| 22 | 10 μM | 10 μmol/L | |
| 23 | 1N HCl | 1 mol/L HCl | |
| 24 | 1N H ₂ SO ₄ | 0.5 mol/L H ₂ SO ₄ | |
| 25 | 4rd edition | 4 th edition | |
| 26 | 15 year experience | 15- year experience | |
| 27 | 18.5 kDa | 18.5 ku, 18 500u or M:18 500 | |
| 28 | 25 g.kg ⁻¹ /d ⁻¹ | 25 g/(kg·d) or 25 g/kg per day | |
| 29 | 6900 | 6 900 | |
| 30 | 1000 rpm | 1 000 r/min | |
| 31 | sec | s | After Arabic numerals |
| 32 | 1 pg L ⁻¹ | 1 pg/L | |
| 33 | 10 kilograms | 10 kg | |
| 34 | 13 000 rpm | 13 000 g | High speed; g should be in italic and suitable conversion. |
| 35 | 1000 g | 1 000 r/min | Low speed. g cannot be used. |
| 36 | Gene bank | GenBank | International classified genetic materials collection bank |
| 37 | Ten L | Ten liters | At the beginning of a sentence |
| 38 | Ten mL | Ten milliliters | At the beginning of a sentence |
| 39 | umol | μmol | |
| 40 | 30 sec | 30 s | |
| 41 | 1 g/dl | 10 g/L | 10-fold conversion |
| 42 | OD ₂₆₀ | A ₂₆₀ | "OD" has been abandoned. |
| 43 | One g/L | One microgram per liter | At the beginning of a sentence |
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| 46 | KM | km | kilometer |
| 47 | CM | cm | centimeter |
| 48 | MM | mm | millimeter |
| 49 | Kg, KG | kg | kilogram |
| 50 | Gm, gr | g | gram |
| 51 | nt | N | newton |
| 52 | l | L | liter |
| 53 | db | dB | decibel |
| 54 | rpm | r/min | rotation per minute |
| 55 | bq | Bq | becquerel, a unit symbol |
| 56 | amp | A | ampere |
| 57 | coul | C | coulomb |
| 58 | HZ | Hz | |
| 59 | w | W | watt |
| 60 | KPa | kPa | kilo-pascal |
| 61 | p | Pa | pascal |
| 62 | ev | EV | volt (electronic unit) |
| 63 | Jonle | J | joule |
| 64 | J/mmol | kJ/mol | kilojoule per mole |
| 65 | 10×10×10cm ³ | 10 cm×10 cm×10 cm | |
| 66 | N·km | KN·m | moment |
| 67 | x±s | mean±SD | In figures, tables or text narration |
| 68 | Mean±SEM | mean±SE | In figures, tables or text narration |
| 69 | im | im | intramuscular injection |
| 70 | iv | iv | intravenous injection |
| 71 | Wang et al | Wang <i>et al.</i> | |
| 72 | EcoRI | EcoRI | <i>Eco</i> in italic and RI in positive. Restriction endonuclease has its prescript form of writing. |
| 73 | Ecoli | <i>E.coli</i> | Bacteria and other biologic terms have their specific expression. |
| 74 | Hp | <i>H pylori</i> | |
| 75 | Iga | Iga | writing form of genes |
| 76 | igA | IgA | writing form of proteins |
| 77 | ~70 kDa | ~70 ku | |

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Cardiac and vascular changes in cirrhosis: Pathogenic mechanisms

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Abstract

Cardiovascular abnormalities accompany both portal hypertension and cirrhosis. These consist of hyperdynamic circulation, defined as reduced mean arterial pressure and systemic vascular resistance, and increased cardiac output. Despite the baseline increased cardiac output, ventricular inotropic and chronotropic responses to stimuli are blunted, a condition known as cirrhotic cardiomyopathy. Both conditions may play an initiating or aggravating pathogenic role in many of the complications of liver failure or portal hypertension including ascites, variceal bleeding, hepatorenal syndrome and increased postoperative mortality after major surgery or liver transplantation. This review briefly examines the major mechanisms that may underlie these cardiovascular abnormalities, concentrating on nitric oxide, endogenous cannabinoids, central neural activation and adrenergic receptor changes. Future work should address the complex interrelationships between these systems.

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Key words: Hyperdynamic circulation; Portal hypertension; Cirrhotic cardiomyopathy; Hemodynamics; Nitric oxide; Endocannabinoid; cGMP

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INTRODUCTION

The cardiovascular system in patients with cirrhosis or

portal hypertension is abnormal. The circulation becomes hyperdynamic, characterized by increased cardiac output and decreased peripheral vascular resistance and arterial pressure. Moreover, despite the increased cardiac output at rest, with stressful stimuli such as hemorrhage, surgery or vasoactive drug administration, the ventricular response is blunted, a condition known as cirrhotic cardiomyopathy. These cardiovascular abnormalities have been suggested to induce or aggravate several complications of cirrhosis such as renal salt and water retention, variceal bleeding, hepatopulmonary syndrome, and increased cardiovascular fragility under stress. Recent reviews have detailed the clinical aspects of hyperdynamic circulation^[1,2] and cirrhotic cardiomyopathy^[3-5]. This review will summarize the recent work on pathogenic mechanisms underlying these two conditions.

HYPERDYNAMIC CIRCULATION

Peripheral vasodilatation is central to hyperdynamic circulation and portal hypertension in cirrhosis. However, the factors directly initiating vasodilatation remain obscure. A hypothesis that has received much attention over the past three decades is the "humoral factor" theory. In cirrhosis, increased intrahepatic resistance induces portosystemic collateral formation, allowing gut-derived humoral substances to directly enter the systemic circulation without detoxification by the liver. The following gut-derived or locally-produced humoral factors have been implicated as possible mediators of peripheral vasodilatation in cirrhosis or portal hypertension.

Endocannabinoids

Endocannabinoids are lipid-like substances that act on two inhibitory G protein-coupled receptors, CB1 and CB2. The vasodilatory effect of endogenous cannabinoids in cirrhosis was first reported in 2001^[6]. Anandamide, an endogenous cannabinoid or endocannabinoid, is increased in monocytes of cirrhotic rats^[6,7], and its receptor CB1 is also upregulated in the vascular endothelium of patients with cirrhosis^[6]. Infusing monocytes isolated from cirrhotic rats into normal rats decreases the mean arterial pressure in the recipients. Furthermore, administering a CB1 receptor antagonist SR141716A to cirrhotic rats increases the total peripheral resistance^[6,7], both studies demonstrated that SR141716A significantly increases the reduced arterial pressure in cirrhosis, and blocks the hypotension induced by the infusion of isolated cirrhotic monocytes into normal rats^[6,7]. Batkai and colleagues also

found that SR141716A decreases mesenteric blood flow and portal venous pressure in cirrhotic rats^[6]. All of these data indicate that the vascular tone in cirrhosis is regulated by CB1 receptors in both the splanchnic and systemic circulations.

Besides vasodilatation, anandamide rapidly and dose-dependently induces apoptosis in primary culture-activated and *in vivo*-activated hepatic stellate cells, with over 70% cell death after 4 h at 25 $\mu\text{mol/L}$ ^[8]. This effect could alter the hepatic sinusoidal microcirculation and enhance the development of portal hypertension that leads to hyperdynamic circulation.

How does cirrhosis leads to increased endocannabinoids? Varga and co-workers found that bacterial endotoxin stimulates endocannabinoid production in cirrhosis^[9]. The upregulation of CB1 receptors in cirrhotic vascular endothelium and thus increased end-organ sensitivity may also enhance endocannabinoid vasodilator tone^[6].

Nitric oxide

NO has been extensively studied. It is now clear that in cirrhosis, changes in NO activity affect different vascular beds in variable ways. In the liver microcirculation, endothelial-constitutive NO synthase (eNOS or NOS3) expression is decreased in a cirrhotic rat model^[10]. Simvastatin enhances hepatic nitric oxide production and decreases the hepatic vascular tone in patients with cirrhosis^[11]. An NO donor^[12] or NOS3 gene transfection^[10], which compensates for the decreased hepatic NOS3 expression, significantly lowers the increased portal pressure in cirrhosis.

In contrast, systemic NO production is increased in cirrhotic patients and animal models^[13-15]. Moreover, normalization of NO production in cirrhotic rats, by achieving normal concentrations of aortic cGMP with small doses of the NOS inhibitor L-NAME, normalizes the decreased peripheral vascular resistance and the increased cardiac output^[16]. *In vitro*, an NO inhibitor reverses the hyporeactivity of blood vessels from cirrhotic rats to vasoconstrictors^[17].

All these results strongly support the hypothesis that increased NO production is a major factor in the peripheral arterial vasodilation of cirrhosis. Agents promoting nitric oxide production include inflammatory cytokines and endotoxin. In that regard, selective intestinal decontamination with norfloxacin partially reverses the hyperdynamic circulatory state in cirrhotic patients, suggesting a role for the endotoxin-NO pathway^[18]. Where does this endotoxin come from in cirrhosis? First, alcohol is a major cause of cirrhosis in Western countries, and alcoholic gastrointestinal mucosal damage^[19], could potentially facilitate transfer of bacteria into the circulation. Second, portosystemic shunting allows gut-derived bacterial endotoxins passage to the systemic circulation. Third, cirrhotic patients with portal hypertension show intestinal structural abnormalities characterized by vascular congestion and edema, which leads to increased intestinal permeability to bacterial toxins^[20]. Fourth, intestinal bacterial overgrowth and bacterial translocation are increased in cirrhosis^[21]. Besides endotoxins, the other possible factors stimulating NO production include

cytokines such as TNF- α , IL-1, IL-6, and IFN- γ ^[22-24]. Among these, TNF- α has been studied the most. Lopez-Talavera *et al* found that anti-TNF- α antibody increases mean arterial pressure and systemic vascular resistance, and decreases cardiac index and portal pressure^[25]. In our 4-week BDL rats, in parallel with increased serum TNF- α , aortic NOS3 expression and serum nitrate/nitrite concentrations were increased^[26].

Although the evidence is strong that the increased NOS activity in cirrhosis plays an important role in hyperdynamic circulation in cirrhosis, it remains obscure which NOS isoform is involved. The majority of previous studies have used a nonspecific NOS inhibitor to diminish NO production. However, a recent study used aminoguanidine, a preferential inhibitor of NOS2 (inducible NOS), and showed that *in vivo*, the hyperdynamic circulation in portal hypertensive rats is reversed^[27]. But in another study aminoguanidine had no *in vitro* effect on the hyporeactivity of aortic rings from cirrhotic rats^[28]. We have recently evaluated the activity of the L-arginine-NO pathway at different levels^[26]. Although NOS2 mRNA was detectable in the cirrhotic aorta, no NOS2 protein was observed in our Western blots. It is unclear why the mRNA was not expressed as a protein. It might have been degraded or not been transcribed. It is also possible that our method of Western blotting did not allow the detection of small amounts of NOS2 protein.

A consistent augmentation in the expression of NOS3 mRNA and protein levels is observed in cirrhotic rats. Because NOS3 can be upregulated by stimuli such as shear stress and mechanical deformation, some have suggested that hyperdynamic circulation is the cause rather than the consequence of the activation of the NO pathway^[14,29,30]. In addition, there may be other reasons for the increased NOS3. Cirrhosis is associated with increased levels of estrogens^[31,32], and these compounds have been shown to upregulate NOS3 activity^[33]. Other factors which may stimulate NOS3 expression need further investigation.

What is the role of another isoform of NOS, neuronal NOS (nNOS or NOS1)? Xu and his colleagues have demonstrated that nNOS expression is significantly increased in rat cirrhotic aortae^[34]. Furthermore, an nNOS-specific inhibitor, 7-nitroindazole (7-NI), significantly decreased the sodium and water retention and normalized the hyperdynamic indices such as cardiac index, mean arterial pressure, and systemic vascular resistance in these rats^[34]. Biecker *et al* also showed that nNOS partially compensates for the absence of eNOS in producing hyperdynamic circulation in eNOS-gene knockout mice^[35]. These data indicate that the nNOS isoform plays a major pathogenic role in hyperdynamic circulation, and perhaps even in renal salt and water retention in cirrhosis.

It seems that endocannabinoids and nitric oxide may both play an important role in hyperdynamic circulation, but what is the relationship between them? The literature remains inconclusive. In a kidney study, Deutsch *et al* found that the vasodilatation of anandamide is NO dependent, because the NOS inhibitor L-NAME completely blocked the vasodilatory effect of anandamide, similar to a CB1 antagonist^[36]. However, another study showed no effect

of L-NAME infusion on the hypotensive effects of anandamide^[7].

Some studies suggest the possible involvement of other humoral vasodilators, but a definitive pathogenic role for any of these substances remains elusive. This list includes: glucagons^[37], prostaglandins^[38], GABA^[39], VIP^[40], bile acids^[41], endotoxin, histamine^[42] and adenosine^[43].

Central neural mechanisms

Although most research has focused on the humoral mediators, in recent years we and others have shown an important mechanistic role of central nervous system (CNS) activation. A decade ago, our laboratory demonstrated that primary afferent denervation by capsaicin reversed the hyperdynamic circulation in rats with cirrhosis or portal hypertension due to portal vein stenosis (PVS)^[44]. What is the relationship between the CNS and hyperdynamic circulation in portal hypertension? Using *c-fos*, an immediate-early gene (whose protein product can be detected by immunohistochemistry as Fos), as a marker of central neuronal activation, we have showed that the brainstem and hypothalamic cardiovascular-regulatory nuclei are activated at the first day after PVS, whereas the hyperdynamic circulation does not start up until 3-5 days after PVS. This time sequence suggests that central neural activation is the initiating signal in the pathogenesis of hyperdynamic circulation.

Subsequently, in portal hypertensive rats, we microinjected *c-fos* antisense oligonucleotide into one of the major cardiovascular-regulatory brainstem nuclei, the nucleus tractus solitarius (NTS), to block local Fos expression. This treatment completely blocked the development of the hyperdynamic circulation, i.e., abnormalities in cardiac output, mean arterial pressure and systemic vascular resistance were completely eliminated^[45]. In normal control rats, *c-fos* antisense oligonucleotides had no effect^[45]. These results indicate that central neural activation is a *sine qua non* for the development of the hyperdynamic circulation in portal hypertension.

The CNS, as the controller of the circulation, presumably would not arbitrarily activate the cardiovascular system without reason. This raises the question of what the initiating signal is? Likely, it is somehow related to the portal hypertension *per se*. Moreover, the exact route of signaling from the periphery to the CNS remains unclear. The aforementioned capsaicin study suggests that primary afferent nerves may be the signaling pathway from the periphery to the CNS^[44]. Our subsequent study showed that capsaicin-treated BDL rats improve the renal function and do not develop ascites^[46]. Moreover, both BDL-cirrhotic and portal hypertensive rats show diminished Fos expression in NTS after capsaicin-induced denervation of the afferent nerves as neonates^[46]. These observations indicate that intact primary afferent innervation is necessary for the central neuronal activation and development or maintenance of hyperdynamic circulation. Additionally, sodium retention and ascites formation is also dependent on either the presence of hyperdynamic circulation or intact afferent innervation, or both. The complex relationship between CNS activation, local or neurohormonal humoral factor stimulation,

and cardiovascular disturbances in cirrhosis/portal hypertension continues to be studied in several labs.

CIRRHOTIC CARDIOMYOPATHY

This syndrome was first described in the late 1960s, although for many years, it was mistakenly attributed to latent or subclinical alcoholic cardiomyopathy^[47-49]. However, studies in human and animal models with nonalcoholic cirrhosis, dating from the mid-1980s showed a similar pattern of increased baseline cardiac output with blunted response to stress^[4]. The clinical features of cirrhotic cardiomyopathy include blunted systolic and diastolic contractile responses to stress, in conjunction with evidence of ventricular hypertrophy or chamber dilatation and electrophysiological abnormalities including prolonged QT interval. Recent studies suggest the presence of cirrhotic cardiomyopathy may contribute to the pathogenesis of hepatorenal syndrome precipitated by spontaneous bacterial peritonitis^[50], acute heart failure after insertion of transjugular intrahepatic portosystemic shunts (TIPS)^[51,52], and increased cardiovascular morbidity and mortality after liver transplantation^[53]. Therefore this syndrome is more than an academic curiosity, but rather an important clinical entity. We herein review possible pathogenic mechanisms reported by our laboratory and others.

Endocannabinoids

Endocannabinoids are known to have a negative inotropic effect on cardiac contractility in both human^[54] and rats^[55]. The plasma level of an endogenous cannabinoid, anandamide, is known to be increased in cirrhosis^[6]. We recently demonstrated a major role for increased local cardiac production of endocannabinoids in cirrhotic cardiomyopathy^[56]. This conclusion is based on the restoration of blunted contractile response of isolated left ventricular papillary muscles from BDL-cirrhotic rats after preincubation with a CB1 antagonist, AM251. Additionally, endocannabinoid reuptake blockers (VDM11 and AM404) enhance the relaxant response of cirrhotic papillary muscle to higher frequencies of contraction in an AM251-sensitive fashion, suggesting an increase in the local production of endocannabinoids acting through CB1 receptors. Other *in vitro* evidence suggest a main neuronal source for the increase in local production of endocannabinoids, as these effects were mostly abolished by pretreatment with the neurotoxin tetrodotoxin^[56].

β -adrenergic signaling

Cardiac-adrenergic signaling is one of the main regulators of cardiac contractility. Adrenergic receptors increase adenylyl cyclase activity through stimulatory G proteins. Increased production of cAMP in turn results in an increase in calcium influx and contractile force mainly through activation of protein kinase A (PKA). We have previously shown that expression and responsiveness of β -adrenergic receptors^[57] as well as its post receptor signaling pathway is blunted in cardiac tissue of cirrhotic rats. Post receptor impairment was found at different levels including content and function of stimulatory Gs-proteins^[58], uncoupling of the β -adrenoceptor-ligand

complex from G protein^[59], and responsiveness of adenylyl cyclase to stimuli^[58,60].

Membrane fluidity

Biochemical and biophysical properties of the cell membrane determines the mobility of membrane-bound protein moieties. This mobility is known as membrane fluidity^[61], which is shown to be an important factor in the function of a number of membrane-bound receptors including β -adrenergic receptors^[62]. We have shown that membrane fluidity in cardiomyocytes from bile duct-ligated rats is decreased in association with an increase in membrane cholesterol content and cholesterol/phospholipid ratio^[58]. Restoration of these abnormalities *in vitro* results in normalization of blunted response of β -adrenergic receptors^[58]. Alterations in membrane fluidity may also play a role in abnormal function of other membrane-bound components in cirrhotic cardiomyocytes including ion channels. The significant decrease in K^+ currents through Ca^{2+} -independent transient outward K^+ channel and the delayed rectifier current reported by Ward *et al* is an example that requires further investigation^[63].

Nitric oxide

Nitric oxide is known to negatively regulate cardiac contractile function. It has been shown to be involved in some types of cardiac dysfunction including ischemic heart disease^[64]. Balligand *et al* have reported that non-selective blockade of NOS augments the contractile response of rat ventricular myocytes to the β -agonist isoproterenol without affecting the baseline contractility^[65]. Whether this effect is mediated by the inhibition of adenylyl cyclase activity by NO^[66] or through the second messenger, cyclic guanosine monophosphate (cGMP), remains to be elucidated. Possible effects of NO on cardiac function in physiological and some pathophysiological states were extensively reviewed previously^[67,68].

As noted previously, cirrhosis is known to be associated with NO overproduction^[29]. Involvement of NO overproduction in the development of cirrhotic cardiomyopathy was first reported in 1996 by Van Obbergh *et al* in the BDL rat. They showed that a nonselective NOS inhibitor, L-NMMA, restored the blunted contractile function of isolated heart from cirrhotic rats while it had no significant effect in control animals^[69]. We have reported a similar effect in isolated left ventricular papillary muscles of cirrhotic rats. Furthermore, we observed that iNOS and not eNOS mRNA and protein expression were significantly increased in the heart of a cirrhotic rat^[22]. Increased levels of cGMP in cirrhotic ventricles and elevated serum and cardiac levels of cytokines like TNF- α suggest a cytokine/iNOS/cGMP pathway for this effect^[22].

Carbon monoxide

Carbon monoxide (CO) is mainly produced in the body through the action of heme oxygenases. These enzymes are responsible for converting heme to biliverdin and CO. Like NO, CO activates soluble guanylate cyclase resulting in increased levels of cGMP^[70,71]. Expression of inducible heme oxygenase (HO-1) mRNA was increased in the right ventricle in a canine model of congestive heart

failure^[72]. We previously reported an increase in mRNA and protein expression of HO-1 in left ventricle of bile duct-ligated rats, which was associated with an increase in left ventricular cGMP levels^[73]. Furthermore, treatment of cirrhotic heart with an HO inhibitor, zinc protoporphyrin IX, restored the elevated cGMP levels^[73]. These findings suggest the involvement of an HO-CO-cGMP pathway in the development of cirrhotic cardiomyopathy.

CONCLUSION

Cardiovascular abnormalities consisting of hyperdynamic circulation and cardiomyopathy are frequent complications in cirrhotic patients and may contribute to significant morbidity and mortality, especially under stressful conditions. Underlying mechanisms of cardiac dysfunction and vascular abnormalities in cirrhosis have been separately explored in recent years, but a number of vasoactive mediator systems may be common to the genesis of both conditions. We believe that central neural activation plays an important initiating role in the genesis of hyperdynamic circulation, eventually leading to an imbalance between the tonic vasodilator vs vasoconstrictor tone, with a predominance of the former. Predominant among these peripheral vasodilator pathways are NO and endocannabinoids. The mechanisms of cirrhotic cardiomyopathy include altered cardiomyocyte plasma membrane physicochemical properties, impairment of β -adrenergic receptor signaling pathways, and overactivity of NO, carbon monoxide and endocannabinoid systems. Considering the undeniable interrelation of these systems, further studies are required to elucidate the complex interactions between these mechanisms.

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Cutaneous manifestations in celiac disease

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Abstract

Celiac disease (CD) is an autoimmune gluten-dependent enteropathy characterized by atrophy of intestinal villi that improves after gluten-free diet (GFD). CD is often associated with extra-intestinal manifestations; among them, several skin diseases are described in CD patients. The present review reports all CD-associated skin manifestations described in the literature and tries to analyze the possible mechanisms involved in this association. The opportunity to evaluate the possible presence of CD in patients affected by skin disorders is discussed.

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Key words: Celiac disease; Dermatological disease; AGA; EmA; TTG

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INTRODUCTION

Celiac disease (CD) can be defined as a chronic immune-mediated gluten-dependent enteropathy, resulting from an inappropriate T-cell-mediated immune response, against ingested gluten in genetically predisposed people^[1]. Epidemiological studies have shown that CD is very common and affects about 1 in 250 people^[2]. CD is a multigenic disorder associated with HLA-DQ2 (DQA1*/DQB1*2) expressed in more than 90% of patients, or HLA-DQ8 (DQA1*0301/DQB1*0302)^[3]. The expression of these molecules is necessary, but not sufficient, to develop the disease^[4]. The immune response to gluten

takes place in two compartments: the lamina propria and the epithelium. While lamina propria CD4 T cells have a recognized role in the pathogenesis of CD, the role of CD8 T cells in the intestinal epithelium is controversial^[5].

CD is characterized by intestinal malabsorption and subtotal or total atrophy of intestinal villi which improves after gluten-free diet (GFD)^[6]. The classic form of CD presents several symptoms such as diarrhea, abdominal pain, weight loss and nutritional deficiencies, particularly of iron, folate, calcium, and vitamin D^[7]. However, there is a large variety of clinical presentations characterized by the presence of extra-intestinal manifestations, including anemia^[8], persistent hypertransaminasemia^[9], osteopenia^[10], neurological^[11], psychiatric and affective disorders^[12-14], features of hyposplenism (Howell-Jolly bodies and thrombocytosis)^[15] and autoimmune diseases^[6]. In the last years, growing evidence has documented the involvement of skin diseases among the extra-intestinal manifestations of CD^[17]. The aim of this review is to report all CD associated skin manifestations described in the English literature and to analyze the possible mechanisms involved in this association.

Dermatitis herpetiformis

Dermatitis herpetiformis (DH) is a well-described entity, presenting as an itchy, chronic, papulovesicular eruption which may leave pigmentation and scarring^[18]. Classically, skin lesions are characterized by a symmetrical eruption on the extensor surfaces of the body such as the knees, elbows, buttocks, and back. DH tends to occur more in the adult male (M/F ratio 2:1) who may also present with the involvement of oral and genital membranes. However this pattern is reversed in children who may simply have purpura over the palmar surfaces. The age of onset varies with geographical location and the incidence is highest in Ireland and Sweden and rare in Asia^[19]. Histology of lesional skin shows micro abscesses consisting of neutrophils and eosinophils within the dermal papillae. Sub-epidermal vesicles and bullae are produced within the lamina lucida as a result of collagen degradation. Furthermore there is an increased number of activated T cells. Direct immunofluorescence of the normal skin shows the pathogenomic granular IgA deposits in the papillary dermis but the exact target antigen is still unknown^[19-21]. These deposits are often associated with C3 to support the suggestion that the complement is activated via the alternative pathway and C5^[22,23]. The activated fraction, C5a, is highly chemotactic for neutrophils and may contribute to the inflammatory change at the papillary tip.

HLA studies in patients, who have DH based on

clinical and immunological criteria, have shown that 85-90% are HLA B8-positive and that there is an even stronger association with HLA DW3 and DRW3. Interestingly, patients with a GSE without DH show a similar high incidence of these antigens^[24]. Specific B cell/macrophage antigens have been noted in patients with DH and the gluten-sensitive enteropathy^[25]. Family studies have shown that these B cell antigens segregated independently of HLA antigens^[26]. One hypothesis is that both HLA and non-HLA disease genes are necessary for the development of lymphoid cell surface receptors that recognize gluten.

Antigluten, antiendomysium, antigliadin and tissue transglutaminase antibodies have been detected in patients with DH^[27-29]. These gluten-specific cells migrate to the gut mucosa where they mediate cytotoxic reactions involving the epithelial cells^[30]. There is an immunogenetic association with HLA DR3 DQW2 (HLA class II alleles DQAI 0501 and DQBI 0201), which is very much more common in Caucasians than in Orientals and may be important for the different incidences of DH in different ethnic populations. Gluten must be present in the diet for the development of DH; however, a genetic susceptibility to gluten has been proposed as an etiological mechanism. This observation could explain why DH is very common in some Asian populations, which have a low daily gluten intake (e.g., 7-8 g/d in Korea) compared with Western populations which have higher daily gluten intake (e.g., 15-20 g/d in Scandinavia)^[19].

As CD and DH may seem to be vastly different, they both share a unique intestinal sensitivity to gluten, with the rash of DH thought to be an external marker of the underlying intestinal sensitivity that is likely to be the result of molecular mimicry between the auto antigen tissue transglutaminase resident in the gut and the skin derived epidermal transglutaminase.

Although patients with DH are unlikely to have gastrointestinal symptoms or features of malabsorption, they have small intestinal histopathologic changes, with either a classical atrophic appearance with complete villous atrophy, or more commonly a more subtle infiltrative pattern with partial or no villous atrophy. When there is a suspicion of DH with a negative skin biopsy but serologic evidence of CD, a skin biopsy should be repeated given the patchy nature of the lesions, and referral to a gastroenterologist for small bowel biopsy should be pursued. Interestingly, topical skin application of gluten-containing products does not produce the rash of DH in susceptible patients; however, oral or rectal introduction of gluten has been found to result in the classic DH rash^[31,32].

The DH rash is often treated with dapsone in conjunction with the gluten withdrawal. In addition to improving the histological changes of the small intestine, the gluten-free diet may also allow a reduction of suppressive medication^[32]. Just as patients with CD are at a higher risk of malignancies such as lymphomas, so are those patients with DH not compliant with GFD, which should motivate patients with DH to follow the diet regardless of the absence of other symptoms^[31].

Linear IgA bullous dermatosis

Linear IgA bullous dermatosis (LABD) is a rare disease

characterized by erythematous papules or groups of small vesicles. The onset may be acute or gradual and the predominant symptom is usually pruritus. Immunoelectron microscopic studies have shown a linear deposition of IgA along the lamina lucida or in the sub-basal lamina area of stratified squamous epithelium^[32]. At the moment, it is considered as a distinct entity from DH and Bullous pemphigoid^[32,33]. The incidence of gluten sensitivity enteropathy (GSE) in LABD has been described in the literature to range from 0% to 24%^[33-36]. The HLA haplotype (A1, B8, DR3) of LABD patients is often that found in classic DH which is in >85% of CD patients^[37,38]. Although the prevalence of CD is low in LABD, some authors have suggested a diagnosis of GSE in these patients^[34-36,39].

Urticaria

Urticaria is an eruption of transient erythematous or edematous swellings of the dermis or sub-cutaneous tissue and is due to a local increase of permeability of capillaries and small venules. Hautekeete *et al*^[40] first described the association between CD and chronic urticaria, although this association is still debated^[41]. Adults with CD also often have an increase of atopic or immunologic disorders^[42,43]. Scala *et al* reported that a GFD eliminated both skin and intestinal symptoms in patients with concomitant CD and chronic urticaria^[44]. The passage of antigens and immune-complex formation may be facilitated by an increased mucosal permeability. Theoretically, this mechanism might cause urticarial lesions and so, by restoring the integrity of the mucosa, a gluten-free diet might resolve skin symptomatology.

Hereditary angioneurotic edema

Hereditary angioneurotic edema (HANE) is characterized by recurrent edematous attacks in the sub-cutis and sub-mucous due to C1-esterase inhibitor (C1-INH) deficiency^[45]. Brickman *et al*^[46,47] described HANE in association with inflammatory bowel diseases (IBD). In 2002, Farkas *et al*^[48] first described the simultaneous occurrence of HANE and CD. The classic activation pathway of the complement system plays a potential role in the immune regulation of both disorders: it is deficient in HANE and gluten is a potent activator of the alternative pathway in CD^[49]. There might also be a genetically determined etiology of both diseases^[50]. Complement testing is justified whenever the GI symptoms of CD persist despite restoration of damaged mucous. Conversely HANE unresponsive to adequate prophylaxis should prompt for complete GI group tests^[48].

Cutaneous vasculitis

CD may give rise to many skin manifestations but vasculitic skin lesions have rarely been encountered^[51,52]. Leucocytoclastic vasculitis is characterized by the involvement of postcapillary venules. The skin is the most common organ involved with the usual clinical appearance of a palpable purpura. In most cases, leucocytoclastic vasculitis is mediated by immunocomplex deposition and the antigen being either exogenous or endogenous^[53]. The coexistence of cutaneous vasculitis and CD might be related to in-

creased intestinal permeability^[54], and immune complexes, originating from exogenous or endogenous antigens, might circulate because of the impaired phagocytic function of reticular endothelium system and be deposited in the skin^[55]. As in inflammatory bowel disease (IBD), exogenous antigens may permeate the damaged CD mucous in larger quantities than normal^[54,56]. This is reflected by significant serum milk and gluten fraction antibody titers^[57,58]. Alternatively, an autoimmune sensitization may result because of the release of endogenous antigens from damaged small bowel mucosa^[59]. Meyers *et al*^[60] described a case of cutaneous vasculitis complicating CD and the remission of skin lesions after the treatment with a strict GFD. Treatment of leucocytoclastic vasculitis has generally been difficult, but corticosteroids and a gluten-free diet may improve the dermatosis in cases associated with CD^[52].

Erythema nodosum

Erythema nodosum (EN) is a chronic panniculitis characterized by inflammation of the fat septa and its etiology seems to involve an allergic or immune complex-mediated reaction to many antigens. Triggers include infections, sarcoidosis, adverse drug reaction, IBD, and lymphomas^[61]. EN generally resolves in 5-8 weeks but if antigenic stimulus persists, the disease may last for a long period^[62]. In 1991, Durand *et al* first observed a causal linkage between CD and EN^[63]. CD can be a triggering factor of EN because an altered intestinal permeability to endogenous or exogenous antigens may provoke the immunologic response. EN is also a common finding in sarcoidosis which frequently coincides with CD^[62,65]. When EN skin lesions are chronically recurrent or persistent, it is advisable to search for an underlying CD even if it is difficult to estimate the true incidence of the coexistence of both diseases.

Erythema elevatum diutinum

Erythema elevatum diutinum (EED) is a rare variation of cutaneous leucocytoclastic vasculitis, originally described in 1894^[66]. Its lesions appear as soft erythematous papule-plaques and become hard nodules mainly symmetrically affecting the backs of the hands and other extensor surfaces overlying the joints^[67]. Ulceration, arthralgia and pain can be other features of this disease^[67,68]. Even if it is presumed to be an immune reaction by bacterial antigens, the etiology of EED is unknown^[69]. EED has been described in association with several other immunological diseases, especially paraproteinemias (i.e., monoclonal IgA gammopathy)^[70]. Tasanen *et al*^[70] detected dermo-epidermal IgA and complement deposits in lesional skin of a patient affected by CD; the histopathology was suggestive of EED^[71]. The EED skin lesions appear to result from an immune complex reaction, as suggested by the association of various infections or autoimmune diseases. Joint pains are often present in patients affected by autoimmune disease such as EED but are also often found in association with CD^[72]. Some authors have therefore suggested that it is important to warrant consideration of underlying CD in at least some patients with EED^[71-73].

Necrolytic migratory erythema

The features of necrolytic migratory erythema (NME) have been reviewed by several authors. Becker *et al*^[74] first described a cutaneous reaction pattern with specific histopathologic features in association with a pancreatic islet cell carcinoma. NME is a term used by Wilkinson^[75] to describe the characteristic skin disease related with glucagonoma. Typically lesions start with figurate areas of erythema followed by erosions mainly over the trunk, perineum, lower extremities and perioral area. Eventual shedding is caused by superficial necrosis^[76]. Most patients have angular cheilitis, stomatitis, glossitis, diffuse alopecia and brittle nails. Gastrointestinal symptoms such as weight loss and diarrhea are common^[77]. Most cases of NME were part of the glucagonoma syndrome^[76-77]. NME occasionally occurs with chronic liver disease or malabsorption with villous atrophy^[78,79]. Kelly *et al*^[80], Goodenberg *et al*^[79] and Thorisdottir *et al*^[81] described the association between CD and NME. The etiology of NME is uncertain, although deficiencies of zinc, amino acids, or essentially fatty acids could play an important role^[77,78,82,83]. It must be stressed that in CD patients with NME, this latter disorder seems to have an excellent response to GFD^[79,81].

Psoriasis

Psoriasis is a chronic, relapsing dermatosis characterized by scaling, erythema, and less commonly pustulation^[84]. It has been demonstrated that psoriasis is an immunological disease with hyperproliferation of T-cell mediated keratocytes^[85]. Recent data indicate that HLA-Cw*0602 may play an important pathogenetic role in the majority of psoriasis patients^[86]. Immune mechanisms have an important role in the pathogenesis of this disease. In particular, an overexpression of T helper cell type 1 (Th1) cytokines and a relative under-expression of Th 2 cytokines have been shown in psoriatic patients^[87]. The treatment is often difficult; all currently available effective remedies are retinoids or immunosuppressant drugs such as steroids or cyclosporin^[84], other than topical treatment^[88], photochemotherapy (PUVA)^[89] and biological treatment such as infliximab or etanercept^[90]. Recent studies have showed an association between CD and psoriasis^[91,92,93] and an improvement of skin lesions after 3-6 mo of GFD, without other pharmacological approaches, was described^[91]. However at present the relationship between CD and psoriasis remains controversial since there are few data on this topic and other authors sustain that this association seems to be coincidental^[94].

In one study, 10 out of 32 (31%) subjects affected by different clinical forms of psoriasis had positive antireticulin antibodies with a variability of titers, related to the extent of the disease^[95]. Subsequently, in a first screening study conducted by Michaelsson *et al*^[96] 16% of 302 subjects with psoriasis had IgA and/or IgG AGA. Only in 1 of 11 IgA AGA-positive patients from this group screened for antibodies to reticulin/EMA was this positive. An increase of EG2-positive eosinophils in duodenal mucosa associated with elevated serum levels of eosinophil cation protein (ECP) has been documented in psoriatic subjects with AGA, but no tendency to higher

ECP values has been detected in those with AGA^[97]. The same authors reported that the increase in the number of eosinophils in the gastrointestinal tract is explained as being part of some unknown process that may be related to the development of skin psoriatic lesions. Cardinali and co-workers^[92] performed a study to verify whether gluten intolerance is more frequent in Mediterranean subjects with psoriasis than in healthy controls. The sera of 39 psoriatic patients and 39 controls were screened for AGA, EMA, antibodies anti-transglutaminase (TgA), ECP, serum total IgE levels and number of eosinophils. The results showed positivity for IgG AGA in two psoriatic patients, one borderline for TgA, and none was positive for EMA or IgA AGA. In these subjects the authors found high serum levels of ECP and IgE, but a normal number of eosinophils; on the contrary eosinophil count and serum IgE levels were within the normal range in the controls. Michaelsson *et al*^[91] evaluated the effect of GFD in 33 AGA positive patients and six AGA negative patients with psoriasis in an open study. Of the 33 AGA-positive patients, 2 had IgA EMA, and at the duodenal biopsy 15 showed an increased number of lymphocytes in the epithelium, but in some this increase was only slight. GFD was for 3 mo. Thirty out of thirty-three patients strictly complied with GFD, after which they showed a significant decrease of psoriatic lesions. This included a significant decrease in the 16 AGA positive patients with normal routine histology in duodenal biopsy. The AGA negative patients did not improve. There was also a significant decrease in the serum of eosinophil cation protein in patients with elevated AGA. In conclusion, the positive effects of GFD were observed not only in patients with an increased number of lymphocytes in the duodenal epithelium, but also in some patients with seemingly normal epithelium^[91,98].

Our group recently reported a case of severe psoriasis in a CD patient, not responding to specific therapies for psoriasis and in whom the regression of skin lesions after GFD was very rapid^[99,100]. The association between psoriasis and CD was subsequently confirmed by Ojetti *et al*^[101]. The authors evaluated the prevalence of CD in patients affected by psoriasis, showing a high frequency of CD (4.34%) in psoriatic patients.

At present the mechanisms implicated in the possible association between CD and psoriasis, and the effect of GFD on psoriatic skin lesions are not known. There are some hypotheses:

Hypothesis 1: Abnormal small intestinal permeability, frequently present both in psoriasis^[102] and in CD patients^[103], could be a triggering factor between CD and psoriasis.

Hypothesis 2: T cells play an important role in the pathogenesis of both psoriasis and CD. An increased number of T cells in the blood, in the dermis and in the epidermis of psoriatic patients has been documented^[87]. In CD patients, gliadin induces a sensitization of T cells^[104] and this may play a role in the pathogenesis of psoriatic skin lesions^[99].

Hypothesis 3: Psoriatic lesions in CD patients could be related to vitamin D deficiency, which is present both in CD^[105] and in psoriasis^[106-107].

Vitiligo disease

Vitiligo is a specific, common, often heritable acquired disorder characterized by well-circumscribed milky white cutaneous macules devoid of identifiable melanocytes^[108]. This disease appears to be more commonly observed in parts of the body exposed to the sun and in darker skin types and may develop at any age. The etiology is complex: there appears to be a certain genetic predisposition and a number of potential precipitating causes (i.e. crisis, illness, physical injury, etc.). Numerous attempts to identify HLA markers have revealed certain ethnic-specific markers: HLA-DR4 (blacks), B13 (Jews) and BW-35 (Yemenites). Theiligore^[108] has proposed three prevailing theories to explain vitiligo: the neural hypothesis, the self-destruct hypothesis and the immune hypothesis. It carries a risk for ocular abnormalities, particularly iritis, and a significant risk for thyroid disease, diabetes mellitus, Addison's disease and pernicious anemia.

The relationship between CD and vitiligo is controversial. Although some authors have described some cases of vitiligo in patients affected by CD^[94,109], a serological screening study for CD in patients with vitiligo did not show any correlation between these two immunological disorders^[110]. This implies that the sporadic associated cases must be considered as coincidental.

Behçet's disease

A history of oral ulceration has been described in 25% of patients with CD and about 2-4% of patients with recurrent oral ulceration also have CD^[111]. Behçet's disease (BD) is characterized by recurrent ulceration of the mouth and genitalia associated with iritis. Males are affected 5-10 times more frequently than females and the onset is usually between the ages of 10 and 30. The oral ulcers usually show only non-specific inflammatory changes, although there may be infiltration of the grossly thickened walls of thrombosed arterioles. Some authors have suggested that BD may be caused by a virus^[112] and antibodies against mucous membrane were demonstrated in 17 of 40 patients^[113] but their significance has not yet been evaluated. Triolo and co-workers^[114] described 3 cases of coexistence of BD and symptomless CD in 11 patients with BD (defined according to International Study Group-ISG criteria). IgA and IgG class AGA and EMA were found in one patient, another two had IgG AGA alone. There is suggestive but as yet inconclusive evidence of a probable association between CD and autoimmune diseases such as BD. It could be supposed that both diseases are likely the result of molecular mimicry between two undefined auto antigens or virus trigger action. In patients affected by BD, it may therefore be important to search for underlying undiagnosed CD, but future studies are needed to shed light on this point.

Oral lichen planus

The associations between CD and recurrent oral ulceration, glossitis, angular cheilitis and burning mouth are common^[111]. Three to six percent of patients with these oral manifestations may have underlying undiagnosed CD^[111,115]. Fortune *et al*^[116] described the erosive type

of oral lichen planus (OLP) in a patient with gluten enteropathy. OLP is not rare and frequently involves the buccal mucosa, gingiva and tongue. In both the diseases, the lesions had a mucosal T-cell infiltrate and the relief from eating a gluten-free diet was surprising. It is not clear whether the oral lesions are a direct manifestation of CD or due to the effect of malabsorption on the rapidly dividing mucosal cells already predisposed to soreness by a pre-existing disease; because palliative medication of chronic ulcerative stomatitis is often ineffective, it is important to detect the underlying nutritional deficiencies for the patient's general well-being also.

Dermatomyositis

Dermatomyositis is a disorder mainly of the skin, muscle and blood vessels in which characteristic erythematous and edematous changes in the skin are associated with muscle weakness and inflammation. An underlying carcinoma is commonly described in adults. Juvenile dermatomyositis (JDM) has a more favorable prognosis for life but functional disability is usually severe. Its pathogenesis is probably due to cell-mediated immunity to muscle antigens, humoral immunity and immune complex deposition^[117].

Some authors have described an association with CD^[118-121]. Both diseases have a strong association with DQA1 0501 heterodimer because a linkage disequilibrium with HLA-DR3 haplotype or alternatively DR5/DR7 heterozygosity in CD^[122,123] DQA1 0501 may lead to the activation of CD4-positive T cells by presentation of a still unidentified peptide that may induce CD or JDM. Evron *et al.*^[124] and Marie *et al.*^[120] have also described the association between dermatomyositis (DM) and polymyositis (PM) and CD in adults. For these authors CD may be included within the spectrum of gastrointestinal manifestations of DM/PM and they showed that GFD may resolve clinical and laboratory abnormalities.

Porphyria

Mustajoki *et al.*^[125] and subsequently Twaddle *et al.*^[126] reported the coincidental diagnosis of CD in porphyrics. Patients with CD and porphyria present particular problems in terms of management^[127]. The porphyrias are a group of rare diseases affecting the enzymes of the heme biosynthetic pathway with subsequent accumulation of porphyrins in the tissues. These substances are photosensitizers and their accumulation in the skin can cause bullae, erosions, increased fragility, scarring, hirsutism and pigmentation on photo-exposed areas. CD is often associated with DH which has some features in common with skin lesions of porphyrias. In one type of porphyria, variegate porphyria (VP), accumulation of porphobilinogen and 5-aminolevulinic acid is associated with gastrointestinal symptoms and an acute neuropsychiatric syndrome. Some drugs such as dapsone are precipitants of these acute attacks^[127]. It is essential that patients with bullous skin lesions are adequately investigated to distinguish VP from DH because the administration of dapsone could have a potentially fatal outcome. Furthermore, symptomatic control of CD (e.g.,

malabsorption, anorexia, calorie restriction) can prevent precipitation of acute attacks of VP and because gluten sensitivity itself may be associated with neurological and psychiatric disorders^[12,15], neuropsychiatric porphyria could be one of the differential diagnoses^[128].

Alopecia areata

Alopecia areata (AA) is a chronic autoimmune disease characterized by non-scarring alopecia. Histologically it is possible to find perifollicular lymphocyte infiltration, pointing to an immunologic etiology such as CD. In both the diseases there is the presence of organ-specific autoantibodies^[109,129-131], T-lymphocyte infiltration at the site of lesion^[132,133], association with HLA genes^[134,135] and possible etiologic importance of viral co-factors^[136,137].

In the general population the prevalence of CD is 1 in 305^[138] and of AA is 1 in 819^[139]. Corazza and co-workers^[140] found that the prevalence of CD in patients with AA was 1 in 85. Although remission and recurrence may be observed during the clinical course of AA^[141], many patients on gluten-free diet showed complete regrowth of scalp and other body hair and no further recurrence of AA at follow-up^[140,142]. The positive effects of gluten-free diet on the pattern of autoimmune conditions, such as AA, associated with CD have been attributed to a normalization of the immune response. This study suggests that AA patients constitute a novel risk group of CD. Because AA may be the only clinical manifestation of CD, some authors suggest GSE serological screening tests in alopecia universalis^[140].

Acquired hypertrichosis lanuginosa

Acquired hypertrichosis lanuginosa (AHL) is a rare disease and there are less than 40 cases in the literature^[143,144]. This type of hypertrichosis is characterized in its extreme by the rapid growth of fine, down-like hair all over the body, occasionally associated with glossites and loss of taste. A lesser degree of hypertrichosis in which lanugo hair occurs only on the face is more common. It is three times more common in females than in males and most commonly associated with malignancy^[145]. Lymphomas, carcinoid tumors, and the Zollinger-Ellison syndrome have also been associated with this pathology^[145,146].

In 1988, Corazza *et al.*^[147] described the only case reported in the literature of acquired hypertrichosis lanuginosa associated with CD. This case report confirms the paraneoplastic features of AHL. CD is often reported as being associated with tumors and can precede clinical presentation of the tumors^[148].

Pyoderma gangrenosum

The pyoderma gangrenosum (PG) is a neutrophilic dermatosis, with destructive, necrotizing and non-infectious ulcers, involving face and inferior limbs. The association between PG and CD has been described in some case reports and the importance of GFD in the resolution of PG has been suggested^[149].

Ichthyosiform dermatoses

The ichthyosiform dermatoses consist of a heterogeneous

group of hereditary disorders, all of which are characterized by the accumulation of large amounts of scale on the cutaneous surface. The earliest reports of ichthyosis have been traced to the Indian and Chinese literatures several hundred years B.C. and a dermatology text by Willian first discussed the problem. Kinetic studies of the epidermis in the major forms of ichthyosis have shown increased germinative cell hyperplasia and increased transit rate through the epidermis in lamellar ichthyosis^[150]. Menni *et al*^[151] first reported a case of ichthyosis revealing CD but the reason for this association is still unknown.

Pellagra

Pellagra is a disease characterized by lesions of the skin usually preceded by prodromal symptoms, especially of digestive and nervous systems. The dermatosis begins as erythema and edema on the back of the hands, with pruritus and burnings, determined especially by exposure to the sun and by localized pressure. In some patients, several days after the onset of erythema, blisters appear; these run together to form bullae and then break. In the second stage, the dermatosis becomes hard and brittle. The skin may look like a goose. The usual sites are the face, neck, dorsal surfaces of the hands, arms, and feet. The cause of pellagra is still not entirely clear. It may arise from a diet deficient in niacin or tryptophan, or more commonly both, and aminoacid imbalance may also play a part^[152]. In 1999, Schattner^[152] described a case of a man with a pellagra-like syndrome due to CD.

Generalized acquired cutis laxa

Cutis laxa (CL) is a heterogeneous group of inherited and acquired (generalized or localized) diseases characterized by looseness of the skin with the development of inelastic folds that gives the affected person a prematurely senile appearance^[153-155]. CL presents a destructive phenomenon of previously apparently normal elastic fibers, with cephalocaudal progression^[156]. It occurs mainly in adults and the etiology is unknown. Immunological abnormality has been postulated to explain the destruction of elastic tissue^[157]. Generalized acquired cutis laxa (GACL) has been reported in association with infectious disorders, drugs and autoimmune diseases^[153,157,159-163]. Many authors have described a dermal deposit of immunoglobulins (IgA) in various diseases, suggesting that destruction of elastic tissue may be immunologically mediated^[159]. Cases of association between GACL and CD have been reported^[159,164]. Bodvarsson *et al*^[164] proposed that dietary intake of glutenin may induce the production of antibodies to elastin and mediate adherence of neutrophils^[165,166]. A cross-reaction is possible between gluten and some components of elastic fibers, since glutenin presents a 22.3% amino acid sequence similar to elastin^[164,167]. Garcia-Patos and co-workers^[163] described a case of GACL associated with CD, evidence of IgA deposits on the dermal elastic fibers and moderate improvement of eruption after treatment with GFD.

Atypical mole syndrome and congenital giant naevus

Montalto *et al*^[168] first described the association between CD, atypical mole syndrome and congenital giant naevus.

Table 1 Skin disorders in celiac disease

| | |
|---|---|
| Dermatitis herpetiformis | Seah <i>et al</i> ^[20] Mann <i>et al</i> ^[25] Katz <i>et al</i> ^[22] Porter <i>et al</i> ^[28] |
| Linear IgA bullous dermatosis | Leonard <i>et al</i> ^[36] Egan <i>et al</i> ^[39] |
| Urticaria | Hautekeete <i>et al</i> ^[40] Scala <i>et al</i> ^[44] |
| Hereditary angioneurotic edema | Farkas <i>et al</i> ^[48] |
| Cutaneous vasculitis | Holdstock and Oleesky ^[52] Jones ^[51] Meyers <i>et al</i> ^[60] |
| Erythema nodosum | Durand <i>et al</i> ^[63] Crieber <i>et al</i> ^[61] Bartyik <i>et al</i> ^[64] |
| Erythema elevatum diutinum | Collin <i>et al</i> ^[72] Rodriguez-Serna <i>et al</i> ^[73] Tasanen <i>et al</i> ^[71] |
| Necrolytic migratory erythema | Goodenberger <i>et al</i> ^[79] Kelly <i>et al</i> ^[80] Thorisdottir <i>et al</i> ^[81] |
| Psoriasis | Michaelsson <i>et al</i> ^[96] Cardinali <i>et al</i> ^[92] Addolorato <i>et al</i> ^[99] Ojetti <i>et al</i> ^[101] Woo <i>et al</i> ^[93] |
| Vitiligo disease | Reunala and Collin ^[110] Collin and Reunala ^[109] |
| Behçet's disease | Triolo <i>et al</i> ^[115] |
| Oral lichen planus | Fortune and Buchanan ^[117] |
| Dermatomyositis | Evron <i>et al</i> ^[125] Buderus <i>et al</i> ^[119] Falcini <i>et al</i> ^[120] Marie <i>et al</i> ^[121] Iannone and Lapadula ^[122] |
| Porphyria | Mustajoki <i>et al</i> ^[126] Twaddle <i>et al</i> ^[127] |
| Alopecia areata | Corazza <i>et al</i> ^[141] Naveh <i>et al</i> ^[143] |
| Acquired hypertrichosis lanuginosa | Corazza <i>et al</i> ^[148] |
| Ichthyosiform dermatoses | Menni <i>et al</i> ^[152] |
| Pellagra | Schattner ^[153] |
| Generalized acquired cutis laxa | Lewis <i>et al</i> ^[160] Garcia-Patos <i>et al</i> ^[165] |
| Atypical mole syndrome and congenital giant nevus | Montalto <i>et al</i> ^[170] |
| Pyoderma gangrenosum | Gasbarrini and Corazza ^[150] |

They reported a case of a patient affected by a rare multiple disorder of the cutaneous pigmentary system, which increased the risk of melanoma, and by CD which is frequently associated with some neoplasms^[169-172]. Some authors have therefore suggested a more careful objective examination in all the patients with CD^[168].

CONCLUSION

CD is an enteropathy associated with various extra-intestinal manifestations, including several skin diseases. Several hypotheses have been proposed regarding the possible mechanisms involved in this association. In particular, an abnormal small intestinal permeability appears to be implicated, which may allow the crossing of endogenous or exogenous antigens and may provoke the immunological response, common immune mechanisms, vascular alterations and, lastly, vitamin and aminoacid deficiency secondary to malabsorption in patients with CD. However at present the data are not homogeneous and most of the evidence for the association between CD

and skin disorders is based on "case-reports", making it difficult to draw definitive conclusions on this topic; future controlled studies are consequently needed to verify the real involvement of the cutaneous district in CD (Table 1). Nevertheless, despite these limitations, the opportunity to investigate the possible presence of CD in some dermatological patients seems at present justified.

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Probiotics and the gastrointestinal tract: Where are we in 2005?

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Abstract

Probiotic agents are live microbes or components of microbes that have a positive effect on the host. They exert their action through interplay with the immune system of the host. Some of this effect is local and some is systemic. The full story is yet to be discovered. Probiotics have a definite positive effect on rotavirus diarrhea, post antibiotic diarrhea and pouchitis. Their exact role in inflammatory bowel disease, irritable bowel syndrome, other forms of infectious diarrhea, and prevention of cancer is yet to be determined. This review summarizes the data about probiotics in these conditions.

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Keywords: Probiotics; Inflammatory bowel disease; Crohn's disease; Lactose intolerance; Antibiotic associated diarrhea; Rotavirus; Pseudomembranous colitis; Clostridium difficile; Irritable bowel syndrome; Toll like receptor 9

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INTRODUCTION

The modern term 'probiotic' was first used by Fuller [1], describing a live microbial feed supplement which beneficially affects the host animal by improving its microbial balance. Later it was demonstrated that heat-inactivated bacteria or fragments of bacterial DNA have positive effects as well. Marteau *et al* [2] in 2002 defined probiotics as 'microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well-

being'. The mechanisms by which probiotics beneficially affect the host are multiple. Probiotics can prevent or ameliorate diarrhea and inflammation through their local effects and/or their effect on the immune system. In the gut, probiotic bacteria are thought to occupy binding sites on the gut mucosa, preventing pathogenic bacteria from adhering to the mucosa [3,4]. Lactobacilli produce proteinaceous compounds, namely bacteriocins, that act as local antibiotics against more pathogenic organisms [5,6] and decrease production of pro-inflammatory cytokines such as IFN- γ , TNF- α and IL-12 [7-9]. Probiotics stimulate IgA production [10]. Lactobacilli produce acetic and lactic acid and inhibit the growth of bacterial pathogens [3]. It has been postulated that probiotics compete with pathogens for nutrients and modify toxins produced by pathogens or toxin receptors found in the gut wall. Rachmilewitz *et al* [11] have shown that specific DNA repeats isolated from probiotics (VSL #3) can attenuate experimental colitis in various animal models. This is true even with inactivated bacteria. By using toll-like receptor 9 (TLR-9) deficient mice, they have proved that TLR9 signaling is essential in mediating the anti-inflammatory effect of probiotics [12].

Prerequisites for probiotics are to be effective and safe. The characteristics of an effective probiotic as defined by Saavedra [11] are resistance to digestion by enteric or pancreatic enzymes, gastric acid and bile, ability to prevent the adherence, establishment and/or replication of pathogens in the gastrointestinal tract.

Examples of probiotic bacteria are members of the Lactobacilli family such as *Lactobacillus rhamnosus* GG, bifidobacteria and the yeast *saccharomyces boulardii*. There are many candidate bacteria which can be qualified as probiotics, but different bacteria have different actions in different disease states, taking into account that some disease states are better treated with a combination of bacteria and that there is an issue of dosing and viable vs. non viable components of the bacteria. Treatment with probiotics is relatively safe, but not risk free. Probiotics are potentially pathogenic [13]. A recent report describes 3 patients with fungemia in whom the probiotic origin was proven by DNA fingerprinting [14]. Reports of infections of probiotic origin emphasize the fact that these patients are usually immunosuppressed with multiple ports of entry, such as venous and urinary catheter.

This article reviews the use of various probiotics in the treatment of infectious diarrhea, inflammatory bowel disease and lactose intolerance.

PROBIOTICS AND DIARRHEAL DISEASE

Probiotics have been proved to be beneficial to four types of diarrhea: rotavirus diarrhea, antibiotic-associated diarrhea, clostridium difficile diarrhea, and traveler's diarrhea.

Trials of probiotics for other infectious diarrhea diseases have not been consistent in terms of efficacy so far. Paton *et al*^[15] have produced toxin binding probiotics in a breakthrough study using a toxin-binding recombinant probiotic for the treatment and prevention of enterotoxigenic *Escherichia coli* diarrhea. If these probiotics can be proved effective, it might open a whole new era in the treatment of different kinds of diarrhea.

ACUTE VIRAL DIARRHEA

One of the established benefits of probiotics is that they are effective in the treatment of children with acute viral enteritis^[16,17]. *Lactobacillus reuteri* can shorten the course of acute diarrhea in infants from 2.5 days to 1.5 days^[18]. *Lactobacillus casei* GG is effective in treating acute diarrhea as well^[19]. Different strains of probiotics exhibit different efficacy. Kaila *et al*^[20] studied different lactic acid bacteria for their effect on the immune response to rotavirus in children with acute rotavirus gastroenteritis and found that *Lactobacillus casei* subsp. *casei* strain GG (LGG) is most effective on disease duration and produces the highest titers of IgA antibodies. It has been postulated that IgA specific Ab confers immunity against future infectious diarrhea, a higher level of such Ab is desired. Allan *et al*^[21] systematically reviewed 23 papers concerning probiotic treatment of infectious diarrhea and concluded that probiotics appear to be a useful adjunct to rehydration therapy in treating acute infectious diarrhea in adults and children.

ANTIBIOTIC-ASSOCIATED DIARRHEA AND PSEUDOMEMBRANOUS COLITIS

Antibiotic-associated diarrhea (AAD) is seen in up to 39% of hospitalized patients treated with antibiotics^[22] and varies from uncomplicated diarrhea to colitis and pseudomembranous colitis. The pathogenic factors include any one or all the followings: use of antibiotics causing changes in the normal gut flora, pathogenic bacteria taking advantage of the situation and causing inflammation and diarrhea, change in bacterial flora causing changes in carbohydrate metabolism and changes in short chain fatty acid metabolism and absorption. The latter is interfered and osmotic diarrhea ensues. The well-known syndrome of pseudomembranous colitis characterized by high levels of toxin forming *Clostridium difficile* may take a fulminate course with a high recurrence and mortality rate. AAD can be prevented or treated with probiotics. A meta-analysis summing the results of nine controlled trials indicates that both *Lactobacilli* and *S. boulardii* are effective in preventing AAD^[23]. Various probiotics have been used to prevent and treat *Clostridium difficile*-associated pseudomembranous colitis, including *S. boulardii*, *Lactobacillus* GG, *B. longum* and *B. longum* with *L. acidophilus*. *S. boulardii* and fecal

transplantation can be used through a naso-enteric tube or as an enema^[24]. *S. boulardii* seems to be a choice in prevention and treatment of recurrent *Clostridium difficile* pseudomembranous colitis^[24].

INFLAMMATORY BOWEL DISEASE

Inflammatory bowel disease (IBD) has a complex etiology. Environmental as well as genetic factors play a role. It has been established that microbes play a crucial role in IBD though search for an infectious agent has been futile so far. It was reported that *Lactobacillus* and bifidobacteria counts are significantly reduced in feces of patients with Crohn's disease compared to those with ulcerative colitis and controls and continuous interplay exists between the immune system and intestinal flora, suggesting that normalization of gut flora is a logical means of treatment^[25,26]. Venturi *et al*^[25] treated 20 ulcerative colitis patients with VSL#3 and it is able to change the gut microflora because the faecal concentrations of *Streptococcus salivarius* ssp. *thermophilus*, *Lactobacilli* and bifidobacteria were significantly increased in all patients, compared to their basal level which remained stable throughout study. As mentioned before, probiotics have local and systemic effects. *Lactobacillus casei* strain GG given to pediatric Crohn's patients results in increased serum titers of IgA^[27]. It was also reported that murine colitis was successfully treated with *Lactococcus lactis* secreting interleukin-10^[28].

Probiotics can achieve and maintain remission, prevent post surgical recurrence of Crohn's disease and pouchitis, and treat pouchitis, but an established role of probiotics exists only in the latter two indications. Gosselink *et al*^[29] treated 39 patients with *L. rhamnosus* GG and proved that it had a better effect on these patients than on those not treated with it. Gionchetti *et al*^[30,32] demonstrated that pouchitis episode was reduced by 30% using VSL#3 in a double blind placebo-controlled study. Mirura *et al*^[31] and Kuisma *et al*^[33] showed that remission of chronic pouchitis can be achieved with antibiotics. *S. boulardii* has no efficacy on ulcerative colitis^[34].

Treatment of active ulcerative colitis has been extensively investigated in clinical trials^[34-37]. All the studies proved that probiotics are effective at least on one of the followings: clinical and endoscopic improvement or decrease in proinflammatory cytokine expression. One additional report described six active refractory patients treated with human probiotic infusions and symptoms were abolished in all within four months^[38]. These data support using probiotics in mild to moderate active disease. Three trials showed probiotics can be used as a maintenance treatment of UC^[39-41].

Clinical trials have also been conducted in Crohn's disease patients^[42-45]. Campieri *et al*^[45] compared mesalazine with VSL#3 in 40 patients and found that endoscopic recurrence was significantly reduced noted in probiotic-treated patients, but *Lactobacillus* GG and *L. Jonhsonii* effect cannot prevent post surgical recurrence of Crohn's disease. Treatment of active Crohn's disease has been assessed in some studies^[46,47], no definite conclusion could be reached due to methodological drawbacks.

LACTOSE MALABSORPTION

Lactase deficiency is a frequent condition causing intolerance to lactose due to maldigestion. Kolars *et al*^[48] and Savaiano *et al*^[49] in 1984 showed that absorption of lactose from yogurt is improved compared with milk, presumably due to digestion of lactose by lactase released from the yogurt microorganisms^[50]. Shermak *et al*^[51] examined the effect of yogurt or milk on symptoms and hydrogen breath concentration in children with lactose intolerance and found that symptoms as well as exhaled hydrogen concentration were lower in children challenged with yogurt.

IRRITABLE BOWEL SYNDROME

Evidence for benefit of probiotic treatment in irritable bowel syndrome (IBS) has not been consistent. Nobaek *et al*^[52] conducted a double-blind, randomized placebo-controlled study in 60 unselected patients with IBS, and found that probiotic treatment resulted in improvement of flatulence only. Recently, O'Mahony *et al*^[53] reported patients taking *B. infantis* had improvement in their symptoms, but no difference was observed between patients treated with *L. salivarius* and those treated with placebo. An interesting finding was that the basal ratio of IL-10/IL-12 was lower in IBS patients than in matched healthy controls. Treatment with *B. infantis* but not *L. salivarius* or placebo led to normalization of the ratio, suggesting that inflammation plays a role in the pathogenesis of IBS.

PROBIOTICS AND CANCER

It is assumed that cancer is the outcome of genetic and environmental conditions. In each individual the interplay between the two is different. Cancer is the endpoint of a series of events. Various studies have shown the effect of probiotics on some of the enzymatic pathways and intermediates assumed to precede cancer^[54-56]. Brady *et al*^[54] have summarized the data concerning the use of probiotics in the prevention of cancer in their review. Other studies reported that the use of probiotics is inversely related with aberrant crypts or tumor development^[55,56]

POTENTIAL BENEFIT OF PROBIOTICS

Treatment of constipation with probiotics has yielded confusing results^[57,58]. Probiotic *E. coli* strain has been found to be effective on collagenous colitis^[59].

CONCLUSION

Probiotics are a large group of microbes. Different microbes have different actions in different situations. Probiotics play a definite role in a number of clinical situations, namely rotavirus diarrhea, post antibiotic diarrhea and pouchitis. Their role in other clinical situations is yet to be defined.

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Macrophage inflammatory protein-2 contributes to liver resection-induced acceleration of hepatic metastatic tumor growth

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Abstract

AIM: To study the role of macrophage inflammatory protein (MIP)-2 in liver resection-induced acceleration of tumor growth in a mouse model of hepatic metastasis.

METHODS: After a 50% hepatectomy, 1×10^5 CT26.WT cells were implanted into the left liver lobe of syngeneic balb/c mice (PHx). Additional animals were treated with a monoclonal antibody (MAB452) neutralizing MIP-2 (PHx+mAB). Non-resected and non-mAB-treated mice (Con) served as controls. After 7 d, tumor angiogenesis and microcirculation as well as cell proliferation, tumor growth, and CXCR-2 expression were analyzed using intravital fluorescence microscopy, histology, immunohistochemistry, and flow cytometry.

RESULTS: Partial hepatectomy increased ($P < 0.05$) the expression of the MIP-2 receptor CXCR-2 on tumor cells when compared with non-resected controls, and markedly accelerated ($P < 0.05$) angiogenesis and metastatic tumor growth. Neutralization of MIP-2 by MAB452 treatment significantly ($P < 0.05$) depressed CXCR-2 expression. Further, the blockade of MIP-2 reduced the angiogenic response ($P < 0.05$) and inhibited tumor growth ($P < 0.05$). Of interest, liver resection-induced hepatocyte proliferation was not effected by anti-MIP-2 treatment.

CONCLUSION: MIP-2 significantly contributes to liver resection-induced acceleration of colorectal CT26.WT hepatic metastasis growth.

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INTRODUCTION

Major liver resection initiates rapid regeneration and growth of the remaining liver to restore the functional hepatic capacity^[1]. The cellular changes which provoke liver regeneration are orchestrated by a complex network of different growth factors, cytokines^[1-3] and chemokines^[4-6]. The latter include macrophage inflammatory protein (MIP)-2, ENA-78, and IL-8, which all may act through the CXCR-2 receptor. The role of this receptor has been shown in that blockade with antibodies or deletion in gene-targeted animals significantly reduced the regeneration after hepatectomy^[4,6]. The role of the individual chemokines in regeneration after hepatectomy, however, may be redundant. The murine CXC chemokine MIP-2 is a functional analog of human IL-8^[7]. Similar to IL-8, it acts as a potent neutrophil chemoattractant, contributing to wound healing^[8] and inflammation^[9]. While blockade of the chemokine receptor CXCR-2 decreases regeneration of the residual liver, neutralization of MIP-2 through the use of anti-MIP-2 antibodies did not affect the process of regeneration as indicated by the unchanged liver weight to body weight (BW) ratios^[4]. These findings indicate that other chemokines may replace or act via the CXCR-2 receptor in case of MIP-2 deletion for liver regeneration^[4,6].

Besides parenchymal regeneration, hepatectomy also accelerates tumor growth in the remaining liver^[10-14] as well as in remote organ sites^[15]. Slooter and co-workers showed that a 70% liver resection provokes excessive growth of liver metastases when compared to non-resected controls. Of interest, the intravenous application of TNF- α was effective to significantly reduce these outgrowths of tumor masses^[11]. In fact, the acceleration of tumor growth seems to be proportional to the volume of the resected

liver tissue^[10,11,13]. The cause of enhanced tumor growth in the liver after hepatectomy is likely to be multifactorial. It has been suggested that, besides mechanisms of liver regeneration, residual “dormant” micrometastases or tumor cells in the remnant liver are stimulated through distinct pro-tumorigenic factors^[13,14]. In addition, a depression of local defense mechanisms due to a decrease of Kupffer cell tumoricidal activity and systemic immune paralysis may also contribute to the enhancement of tumor growth^[16]. The role of chemokines, such as MIP-2, in the hepatectomy-induced acceleration of tumor growth, however, has not been elucidated yet.

By use of a murine colorectal hepatic metastasis model, we studied whether the accelerated metastatic growth after hepatectomy is mediated by the CXC chemokine MIP-2, and whether the inhibition of tumor growth by blockade of MIP-2 is associated with a downregulation of angiogenesis and tumor blood vessel formation.

MATERIALS AND METHODS

Experiments were performed after approval by the local governmental ethic committee, and were in accordance with the UKCCCR Guidelines for the Welfare of Animals in Experimental Neoplasia (*Br J Cancer* 1998; 77: 1-10) and the Interdisciplinary Principles and Guidelines for the Use of Animals in Research (New York Academy of Sciences Ad Hoc Committee on Animal Research, NY, USA).

Tumor cell line and culture conditions

The CT26 cell line is an *N*-nitroso-*N*-methylurethane-induced undifferentiated adenocarcinoma of the colon, syngeneic with the BALB/c mouse. For our studies, the CT26.WT cell line (ATCC CRL-2638[®], LGC Promochem GmbH, Wesel, Germany) was grown in cell culture as monolayers in RPMI-1640 medium with 2 mmol/L L-glutamine (Sigma Aldrich Chemie GmbH, Taufkirchen, Germany) supplemented with 10% fetal calf serum (FCS Gold, PAA Laboratories GmbH, Cölbe, Germany), 100 U/mL penicillin and 100 µg/mL streptomycin (PAA Laboratories GmbH). The cells were incubated at 37°C in a humidified atmosphere containing 50 mL/L CO₂ in air. Only cells of the first two serial passages after cryo-storage were used. At the day of implantation, CT26.WT cells were harvested from sub-confluent cultures (70%-85%) by trypsinization (0.5 g/L trypsin and 0.2 g/L EDTA, PAA Laboratories GmbH), and washed twice in phosphate-buffered saline solution (PBS, PAA Laboratories GmbH) before being resuspended in PBS at 1×10⁷ cells/L.

Flow cytometric analysis of CT26.WT cells

FACScan (Becton Dickinson, Mountain View, CA, USA) analysis was performed to assess the expression of the chemokine receptor CXCR-2 of the CT26.WT cells. After trypsinization, the cells were fixed in 1 mL Cytifix/Cytoperm (BD Biosciences, Heidelberg, Germany) for 20 min at 4°C, washed twice with Perm Wash (BD Biosciences) and incubated at room temperature for 40 min with a polyclonal rabbit anti-mouse CXCR-2 antibody (Santa Cruz, Heidelberg, Germany) or an

isotype-matched control antibody (Dianova, Hamburg, Germany). A goat anti-rabbit Cy3-conjugated antibody (1:25; Dianova) was used for fluorescence labeling. To remove excess of antibody, cells were washed again and were then maintained in 10 g/L paraformaldehyde in PBS. Flow cytometry was performed within the next 3 h. The FACScan flow cytometer was calibrated with fluorescent standard microbeads (CaliBRITE Beads, BD Biosciences) for accurate instrument setting. Tumor cells were selectively analyzed for their fluorescence properties using the CellQuest data handling program (BD Biosciences) with the assessment of 5 000 events per sample.

Hepatectomy and tumor cell implantation

Eight to twelve weeks old female BALB/c mice with a BW of 18-20 g, kept in a temperature and humidity controlled 12-h light/dark cycle environment, were used. The animals were anesthetized by intraperitoneal injection of 25 mg/kg BW xylazin hydrochloride (Rompun[®], Bayer, Leverkusen, Germany) and 125 mg/kg BW ketamine hydrochloride (Ketavet[®], Pharmacia GmbH, Erlangen, Germany). The anesthetized animals were placed in supine position. After laparotomy through a midline incision, a 50% hepatectomy was performed. After ligation of the supplying and draining blood vessels with PDS 5-0 (Ethicon, Johnson & Johnson Company, Norderstedt, Germany), the right lateral lobes (ventral and dorsal), the right medial lobe and a part of the left medial lobe were resected. The weight of the resected tissue was 0.35 ± 0.01 g. The procedure of intrahepatic implantation of CT26.WT tumor cells was performed as described previously^[17]. The left liver lobe was gently mobilized and 1×10⁵ tumor cells in 10 µL PBS were implanted under the capsule of the lower surface using a 25-µL syringe with a 32-G needle (Gastight[®] #1702, Hamilton Bonaduz AG, Bonaduz, Switzerland). The puncture site was sealed with acrylic glue (Histoacryl[®], B. Braun, Aesculap AG, Tuttlingen, Germany), and the left liver lobe was repositioned anatomically into the peritoneal cavity. The abdominal wall was closed by a one layer technique with a polypropylene suture (Prolene[®] 5-0).

Intravital fluorescence microscopy

At d 7 after tumor cell implantation, the animals were again anesthetized and laparotomized. The left liver lobe with the tumor was exteriorized and placed on an adjustable stage, so that the lower surface of the lobe was positioned horizontally to the microscope. This guaranteed adequate focus levels for the *in vivo* fluorescence microscopy procedure. The surface of the lobe was covered with a cover slip to avoid drying of the tissue and influences of the ambient oxygen^[17]. *In vivo* fluorescence microscopy was performed by epi-illumination technique using a modified Zeiss Axiotech microscope (Zeiss, Oberkochen, Germany)^[18]. Microscopic images were monitored by a charge-coupled device video camera (FK 6990, Prospective Measurements Inc., San Diego, CA, USA) and were transferred to a video system (VO-5800 PS, Sony, München, Germany) for subsequent off-line analysis. Angioarchitecture and microvascular perfusion were analyzed after intravenous contrast enhancement with sodium fluorescein (2 µmol/kg; Merck, Darmstadt,

Germany) using blue light epi-illumination (450 to 490 nm > 520nm, excitation and emission wavelengths).

Microcirculation analysis

Assessment of hepatic microcirculatory parameters was performed off-line by frame-to-frame analysis of the videotaped images using a computer-assisted image analysis system (CapImage; Zeintl, Heidelberg, Germany). Sinusoidal, capillary and venular diameters (μm) were measured perpendicularly to the vessel path. Sinusoidal density within the liver and capillary density within the tumors (cm/cm^2) were determined by measuring the length of red cell perfused capillary per area of observation. The number of venules draining the blood flow from the tumor was counted and was given per tumor margin. The angiogenic front neighboring the tumor margin, characterized by newly developed, chaotically arranged capillaries, was measured in mm^2 surface area.

Morphological examinations

At the end of the experiment, the left liver lobe was harvested and cut into slices with a diameter of 1 mm using a Tissue Slicer (McIlwain Tissue Shopper, Saur Laborbedarf, Reutlingen, Germany). Slices were documented with the use of a stereo-microscope (Leica M651, Leica Microsystems AG, Heerbrugg, Switzerland) and a video system, and the size of the tumor was measured with CapImage according to: tumor volume (mm^3) = $4/3\pi \times a/2 \times b/2 \times c/2$ (a , b , and c represent the three perpendicularly orientated diameters of the tumor).

Histochemistry and immunohistochemistry

For light microscopy, formalin-fixed biopsies were embedded in paraffin. Five micrometer thick sections were cut and stained with hematoxylin and eosin for routine histology according to standard procedures.

Proliferating cell nuclear antigen (PCNA) staining was performed using indirect immunoperoxidase techniques. Therefore, deparaffinized sections were incubated with 30 mL/L H_2O_2 to block endogenous peroxidases and with 20 mL/L goat normal serum for blocking unspecific binding sites. A monoclonal mouse anti-pan PCNA antibody (PC10, 1:50; DakoCytomation, Hamburg, Germany) was used as primary antibody. A biotinylated goat anti-mouse rabbit IgG antibody was used as secondary antibody for streptavidin-biotin-complex peroxidase staining (1:200, LSAB 2 System HRP, DakoCytomation). 3,3'-Diaminobenzidine (DakoCytomation) was used as a chromogen. Sections were counterstained with hemalaun according to Mayer and examined by light microscopy.

For CXCR-2 immunohistochemistry tumor slices were embedded in Tissue Freezing Medium (Jung; Leica Microsystems, Nussloch, Germany), snap-frozen in liquid nitrogen and stored at -80°C . Five micrometer thick cryostat sections were cut, fixed in 4°C cold acetone for 5 s followed by fixation in 16 g/L formaldehyde for 10 min and blocked with 2% normal donkey serum. Tissue sections were then incubated with the polyclonal rabbit anti-mouse CXCR-2 antibody (1:100; Santa Cruz, CA, USA). A donkey anti-rabbit IgG HRP conjugated antibody (1:500; Amersham, Freiburg, Germany) was used

as secondary antibody. 3,3'-Diaminobenzidine was used as chromogen. Sections were counterstained with hemalaun according to Mayer and examined by light microscopy. As a negative control, additional slices from each specimen were exposed to appropriate IgG isotype-matched antibody (Sigma Aldrich Chemie GmbH) in place of primary antibody under the same conditions to determine the specificity of antibody binding. All of the control stainings were found negative.

Experimental protocol

A total of 21 animals received tumor cell implantation and were assigned to three different groups. Animals without liver resection and without further treatment received tumor cell implantation in the left liver lobe and served as controls (Con; $n=7$). In the additional two groups ($n=7$ each), a 50% hepatectomy was performed before the tumor cells were implanted into the left liver lobe. One group of animals (PHx+mAB) received 1 mg/kg BW of the monoclonal rat anti-mouse MIP-2 antibody (MAB452, R&D Systems, Wiesbaden, Germany) at the day of operation and every second day thereafter. Untreated animals with 50% liver resection served as hepatectomy controls (PHx). Seven days after tumor cell implantation and treatment, all animals underwent intravital microscopic examination and the liver and tumor tissue were harvested for histology and immunohistochemistry.

Statistical analysis

All values were expressed as mean \pm SE. After proving the assumption of normality and homogeneity of variance across groups, differences between groups were calculated by a one-way analysis of variance (ANOVA) followed by a *post hoc* Student-Newman-Keuls test, including correction of the alpha error to compensate for multiple comparisons. Overall statistical significance was set at $P<0.05$. Statistical analysis was performed with the use of the software package SigmaStat (SPSS Inc., Chicago, IL, USA).

RESULTS

Tumor growth

Tumor cell implantation, hepatectomy and treatment with the anti-mouse MIP-2 antibody did not affect the general conditions of the animals. All animals had an uneventful postoperative recovery, and, regardless of the group, animals did not show significant changes of BW during the 7-d experimental period. Intravital fluorescence microscopy at d 7 after tumor cell implantation showed that hepatectomy (PHx) provoked a significant ($P<0.05$) acceleration of tumor growth, as indicated by an increased tumor area at the surface of the liver when compared with that of controls (Figure 1). Analysis of the tumor volume confirmed the significantly ($P<0.05$) increased tumor growth, demonstrating a tumor mass which was 18-fold greater than that of controls (Figure 1). Treatment with the MIP-2 antibody (PHx+mAB) was capable of significantly ($P<0.05$) reducing the hepatectomy-induced acceleration of tumor growth, resulting in a tumor volume which was only 6-fold of that of non-resected controls (Figure 1).

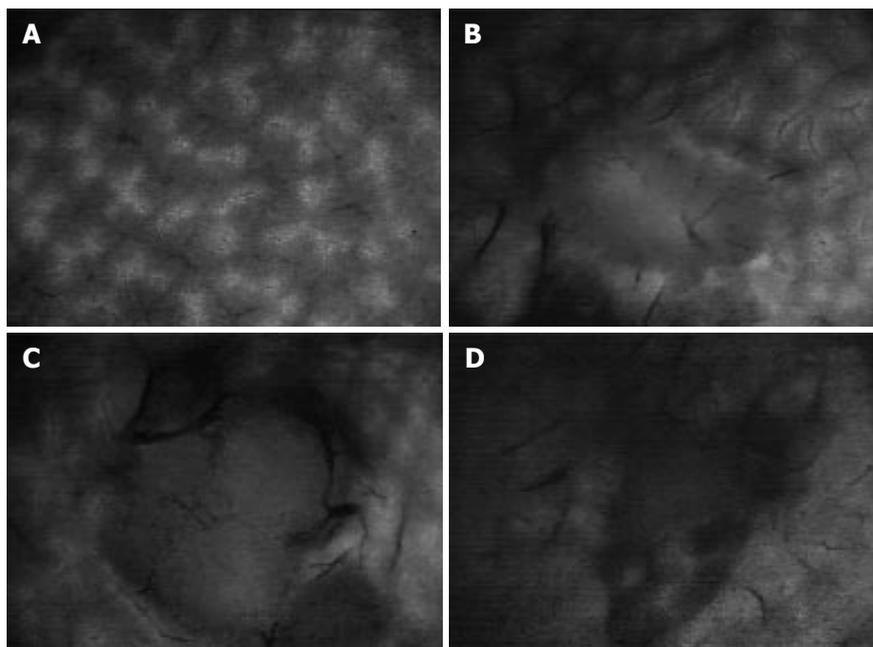
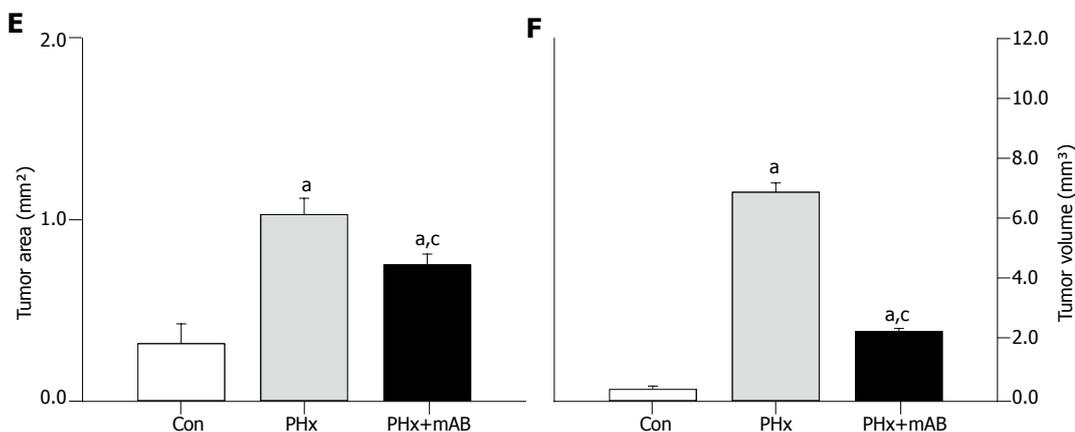


Figure 1 Intravital fluorescence microscopy at d 7 after tumor cell implantation. **A:** Displays the normal liver microvasculature; **B:** shows the microvasculature of the tumor of a non-resected control animal (Con); **C:** displays a 7-day tumor and its microvasculature of an animal which underwent hepatectomy (PHx); **D:** shows the tumor microvasculature after additional anti-MIP-2 treatment (PHx+mAB). Quantitative analysis of tumor area (**E**) and tumor volume (**F**) showed that hepatectomy (PHx) markedly accelerated tumor growth when compared with controls (Con), and that additional anti-MIP-2 treatment (PHx+mAB) was capable of significantly reducing this liver resection-induced tumor growth. Mean \pm SE; ^a $P < 0.05$ vs Con; ^c $P < 0.05$ vs PHx. Magnifications (**A-D**) $\times 16$.



Angiogenesis and microangioarchitecture

Intravital fluorescence microscopy revealed that normal liver presents with a regularly arranged architecture of sinusoids and homogeneous staining of parenchymal cell nuclei (Figure 1A). In contrast, the center of the tumor showed a density of capillaries which was markedly lower than that of the sinusoids in the normal liver (Figure 1 and 2). The margin of the tumor showed an angiogenic front with newly developed, chaotically arranged capillaries (Figure 2) and large draining venules (Figure 3). In control animals, the implantation of CT26.WT cells induced an area of the tumor surrounding angiogenic front of 0.33 mm² at day 7 (Figure 2). Hepatectomy (PHx) significantly increased the area of the angiogenic front by 3-fold ($P < 0.05$) when compared with that of controls. Blockade of MIP-2 (PHx+mAB) was capable of inhibiting angiogenesis, as indicated by a significantly ($P < 0.05$) reduced area at the tumor margin which showed new blood vessel development (Figure 2D).

Because angiogenesis is regularly associated with vasodilation, we analyzed the capillary diameters within the angiogenic front. Within the tumor margin, hepatectomy (PHx) induced a significant dilation of the newly formed capillaries ($P < 0.05$) when compared with that of controls.

Additional anti-MIP-2 treatment (PHx+mAB) further pronounced ($P < 0.05$) this dilatatory effect (Figure 2E). Of interest, the enlargement of the newly formed capillaries within the angiogenic front after hepatectomy and MIP-2 treatment was a homogeneous response, as indicated by the fact that the coefficient of variance of capillary diameters did not differ when compared with untreated controls (Figure 2F). Further, hepatectomy (PHx) also significantly ($P < 0.05$) increased the density of venules draining the tumor, as indicated by a 6.5-fold increase of the number of venules within the tumor margin when compared to controls (Figure 3). These venules were also found enlarged in diameter (Figure 3E), however, without increased heterogeneity when compared with controls (Figure 3F). Interestingly, blockade of MIP-2 significantly ($P < 0.05$) reduced the number of draining tumor venules when compared with animals undergoing only hepatectomy (Figure 3D).

Tumor morphology

Hematoxylin-eosin-stained sections revealed solid growth of the colorectal CT26.WT hepatic metastasis in all the animals (Figure 4). Satellite metastases were not observed in the left liver lobe. In the control group, tumors were

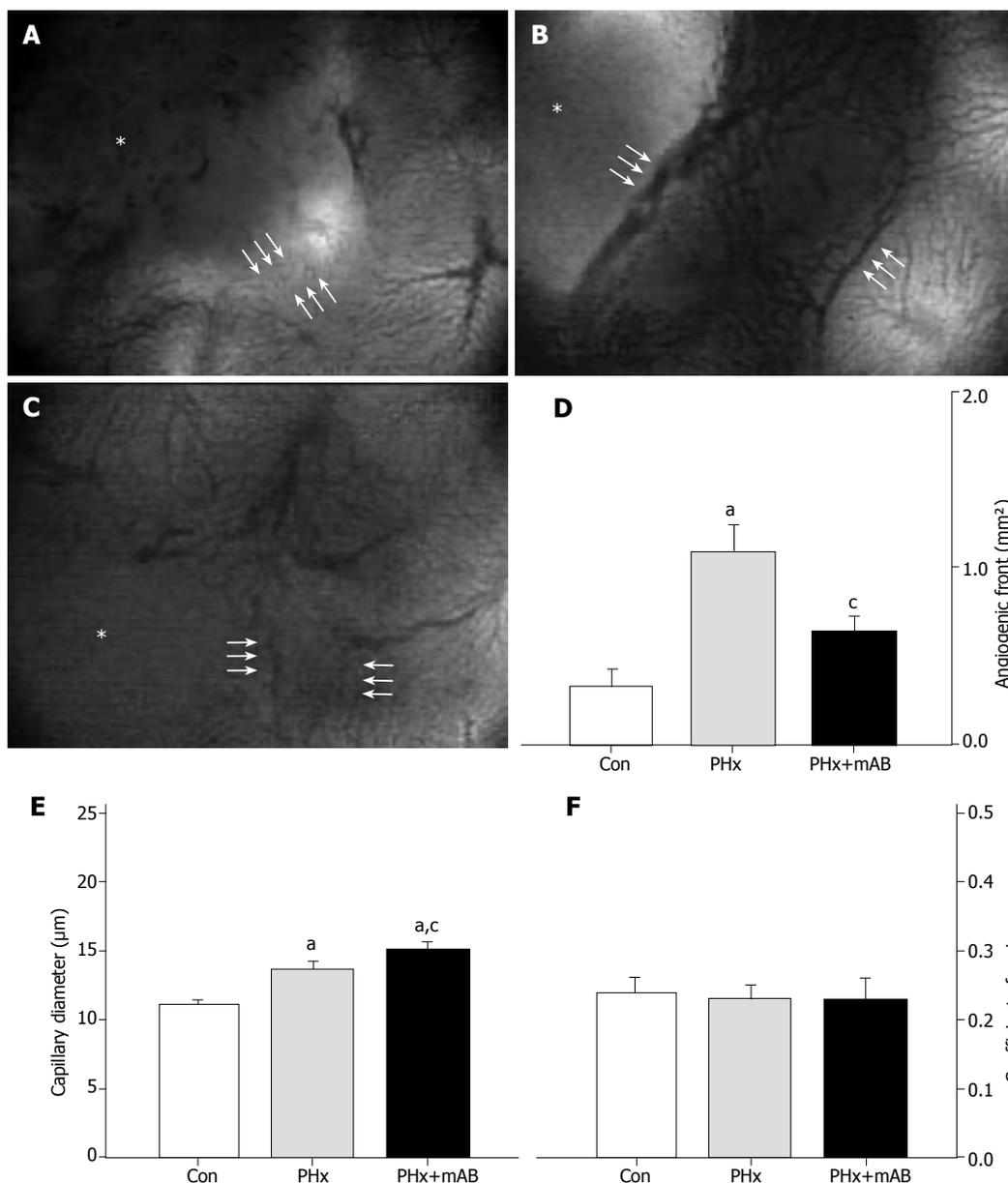


Figure 2 Analysis of the size of the angiogenic front (area between the triple arrows) at the margin of tumors (asterisks) in control mice (A, Con), after hepatectomy (B, PHx) and after hepatectomy and additional anti-MIP-2 treatment (C, PHx+mAB). Quantitative analysis of the area of the angiogenic front (D) indicated a significant increase after hepatectomy (PHx) when compared with that of controls (Con). Additional anti-MIP-2 treatment (PHx+mAB) was capable of reducing the angiogenic process. Further, hepatectomy (PHx) significantly increased capillary dilation within the angiogenic front (E) compared to controls (Con), which was even more pronounced after additional anti-MIP-2 treatment (PHx+mAB). The heterogeneity of capillary diameters, as given by the coefficient of variance, was not affected in either of the treatment groups (F). Mean \pm SE; ^a $P < 0.05$ vs Con; ^c $P < 0.05$ vs PHx. Magnifications (A-C) $\times 40$.

round in nature with a homogeneous appearance, however, with aggressive growth characteristics (Figures 4A and B). Hepatectomy (PHx) accelerated tumor growth, resulting in enlarged tumors with a dense arrangement of tumor cells in the center (Figures 4C and D). In two of the seven animals, lung metastases could be detected macroscopically, although no additional metastases could be found in the abdominal cavity. In contrast, tumors of animals, which additionally were treated with anti-MIP-2 showed central cell death via either necrotic or apoptotic pathways (Figures 4E and F). In this group tumor growth at distant sites was not observed.

In vitro FACScan analysis and CXCR-2 expression *in vivo*

FACScan analysis showed that 98.8% of the CT26.WT cells were CXCR-2 receptor positive *in vitro* (Figure 5). *In vivo*, CXCR-2 immunohistochemistry revealed that ~40% of the tumor cells were receptor-positive. The majority of these CXCR-2 expressing cells were located within the tumor margin (Figure 6). Quantitative analysis of CXCR-2 expression indicated that hepatectomy (PHx)

significantly increased ($P < 0.05$) CXCR-2 expression to ~60% of tumor cells (Figure 6). Blockade of MIP-2 (PHx+mAB) not only abolished the hepatectomy-induced increase of CXCR-2 expression, but further reduced ($P < 0.05$) CXCR-2 expression on tumor cells to only ~10% (Figure 6).

Proliferation of tumor cells and normal liver

PCNA as an indicator of cell proliferation showed that >90% of tumor cells displayed strong PCNA staining (Figure 7). By this, the tumor sharply demarcated from the surrounding liver tissue. Notably, neutralization of MIP-2 (PHx+mAB) decreased the rate of PCNA-positive cells to ~60% (Figures 7C and D), indicating a reduction of proliferation by deletion of MIP-2 function. Normal liver tissue only occasionally showed single PCNA-positive cells (Figure 8). Hepatectomy (PHx) resulted in a significant ($P < 0.05$) increase of the number of PCNA-stained cells when compared with that of non-resected controls (Figure 8). Of interest, additional blockade of MIP-2 induced

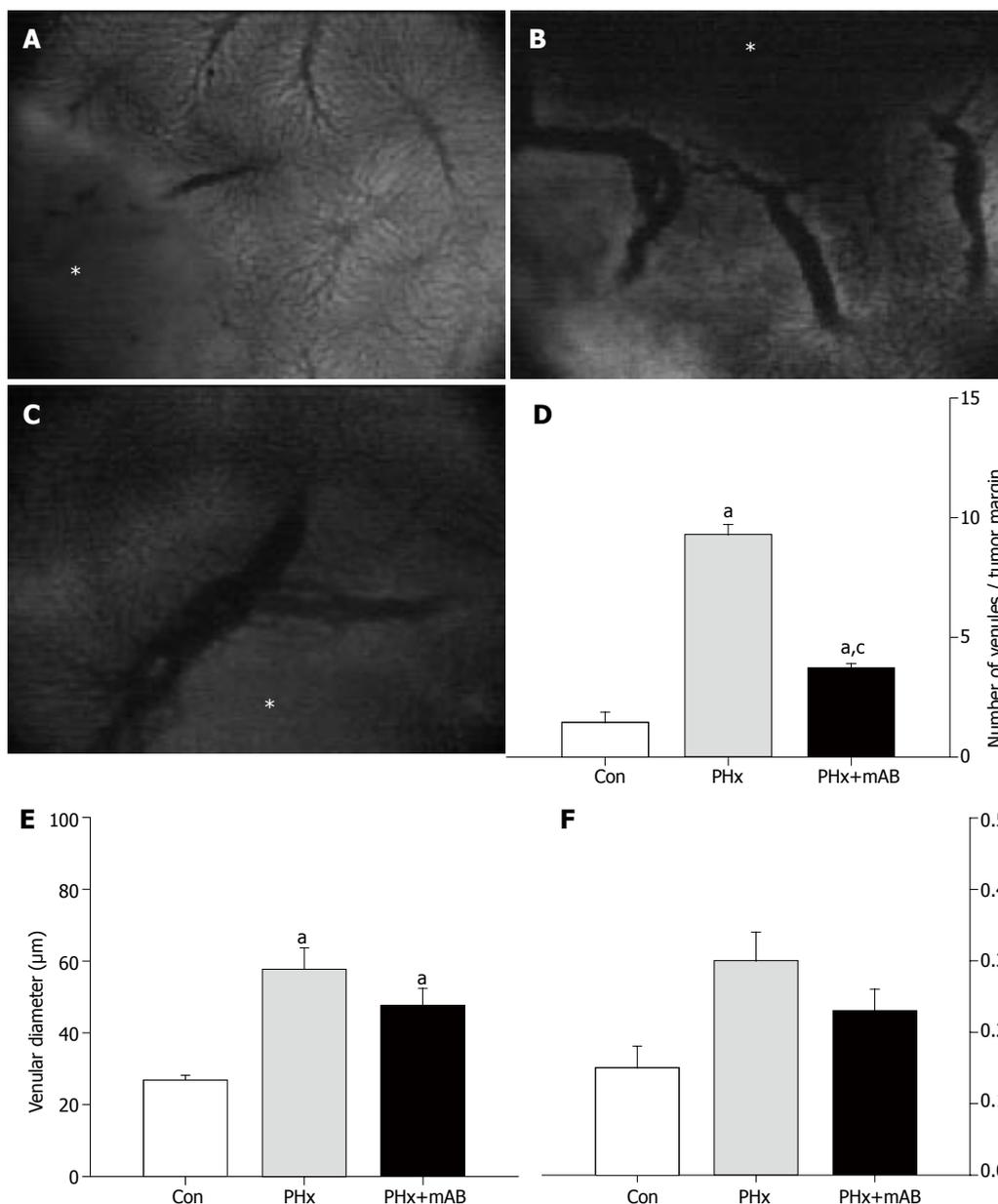


Figure 3 Analysis of the number of venules within the margin of tumors (A-C, asterisk) of control mice (A, Con), after hepatectomy (B, PHx) and after hepatectomy and additional anti-MIP-2 treatment (C, PHx+mAB). Quantitative analysis showed that hepatectomy significantly increases the number of venules (D), and causes a marked dilation of the draining venules within the margin of the tumors (E). Additional anti-MIP-2 treatment was capable of significantly reducing the liver resection-induced increase of the number of tumor venules (D). The heterogeneity of venular diameters, as given by the coefficient of variance, was not affected in either of the treatment groups (F). Mean \pm SE; ^a $P < 0.05$ vs Con; ^c $P < 0.05$ vs PHx. Magnifications (A-C) $\times 40$.

a further increase ($P < 0.05$) of the fraction of PCNA-positive cells in the normal liver (Figure 8).

DISCUSSION

The major findings of the present study are that (1) MIP-2 significantly promotes liver resection-induced expression of the chemokine receptor CXCR-2, and (2) blockade of MIP-2 blunts the augmentation of angiogenesis and metastatic tumor growth after hepatectomy. This indicates for the first time a significant role of this chemokine in the acceleration of tumor growth after hepatectomy. The rodent liver fully regenerates within 8 d after resection^[19,20]. The setpoint for growth regulation is the ratio between liver mass and body mass rather than liver mass *per se*^[1]. Using a 70% liver resection model in mice, Drixler *et al.* demonstrated that liver regeneration is accompanied by intrahepatic angiogenesis^[20]. Liver resection has also been shown to affect the immune system in the early phase of liver regeneration^[3]. The MIP-2-CXCR-2 system is thought

to be involved in the hepatic regenerative processes after extended resection^[4,6]. While blockade of the chemokine receptor CXCR-2 decreases regeneration of the residual liver, selective neutralization of MIP-2 through the use of anti-MIP-2 antibodies is not affecting the liver-to-BW ratio^[4]. Of interest, CXCR-2 seems to be pivotal, because other chemokines may replace or act via the CXCR-2 receptor in case of MIP-2 deletion for liver regeneration^[4,6].

After resection of colorectal liver metastases, diseases recur in over two-thirds of the patients^[21]. This observation indicates that undetected residual microscopic tumors remain in the majority of patients undergoing curative hepatectomy^[22]. The locally released factors which trigger the process of regeneration after hepatectomy may even augment the outgrowth of these residual microtumors, thus, additionally contributing to the high rate of recurrences. This view is supported by experimental studies, demonstrating that liver resection enhances tumor growth during regeneration^[11,13,14,16]. Using different rat hepatectomy models, these authors suggested

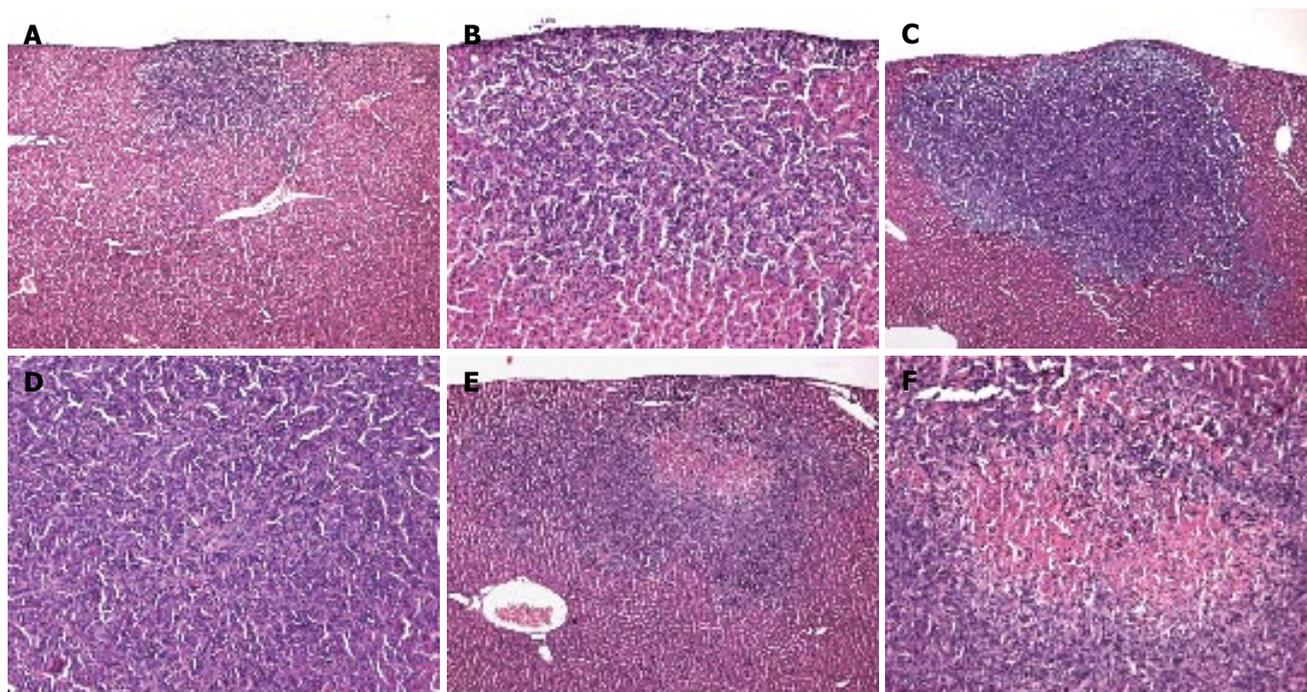


Figure 4 Hematoxylin-eosin-staining of tumors of a control mouse (A and B), a mouse which underwent hepatectomy (PHx) (C and D), and a mouse which additionally received anti-MIP-2 treatment (E and F). Sections revealed solid growth of the colorectal CT26.WT hepatic metastasis in all the animals. Tumors were round in nature, however, showed aggressive growth characteristics. Anti-MIP-2 treatment provoked an area of necrosis within the tumor center (E and F). Magnifications (A, C and E) $\times 18$, (B, D and F) $\times 88$.

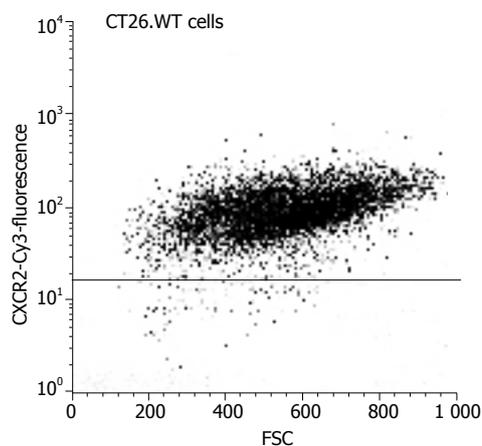


Figure 5 FACSscan analysis of CT26.WT cells, demonstrating positive staining and thus expression of the chemokine receptor CXCR-2 by almost all of the tumors cells studied.

that immunoparalysis with lack of Kupffer cell function and TNF- α response as well as overproduction of growth factors, such as TGF- α , TGF- β and bFGF, contribute to the acceleration of tumor growth.

For the first time, we have herein demonstrated that MIP-2 is involved in hepatectomy-induced metastatic tumor growth. So far, little is known about the role of MIP-2 in tumorigenesis. A recent study indicated that the reduction of fibrosarcoma growth by inhibiting lymphotoxin- β receptor can be reconstituted through co-transfection with MIP-2^[25]. The mechanism how MIP-2 is involved in the herein observed liver resection-induced acceleration of tumor growth seems to be the

pro-proliferative action of this chemokine, because neutralization of MIP-2 significantly reduced PCNA expression on the tumor cells. Of interest, regeneration of the normal liver was not affected by the anti-MIP-2 treatment, inasmuch as hepatocyte PCNA expression after resection was found even increased when compared with that of untreated hepatectomized controls. This is in line with other experimental studies, which demonstrate that blockade of MIP-2 alone did not affect the process of regeneration^[4].

In the present study, we have shown that *in vitro* almost all CT26.WT cells expressed the CXC receptor CXCR-2. *In vivo*, approximately 40% of the cells of non-treated CT26 tumors showed positive CXCR-2 staining 7 days after tumor cell implantation. The reduction of the number of CXCR-2 positive cells is probably due to the fact that solid tumors *in vivo* consist besides the CT26.WT clone of a variety of other cells, including endothelia, fibroblasts and stromal cells, which do not necessarily express CXCR-2. Furthermore, we have demonstrated for the first time that over a 7-d period partial hepatectomy increased the expression of CXCR-2 to ~60%. Because receptor expression seems to be required for the proliferative action of chemokines on tumors^[24], the increased *in vivo* expression of CXCR-2 may represent the mechanism of action of the MIP-2-associated acceleration of tumor growth after hepatectomy. The neutralization of MIP-2 by MAB452 significantly reduced CXCR-2 expression within the tumor. Although we cannot exclude that neutralization of MIP-2 directly alters the CXCR-2 expression of tumor cells, the histological findings may additionally suggest a relative increase of CXCR-2-negative stromal cells.

The progressive growth of a malignant solid tumor depends on the development of new blood vessels. The

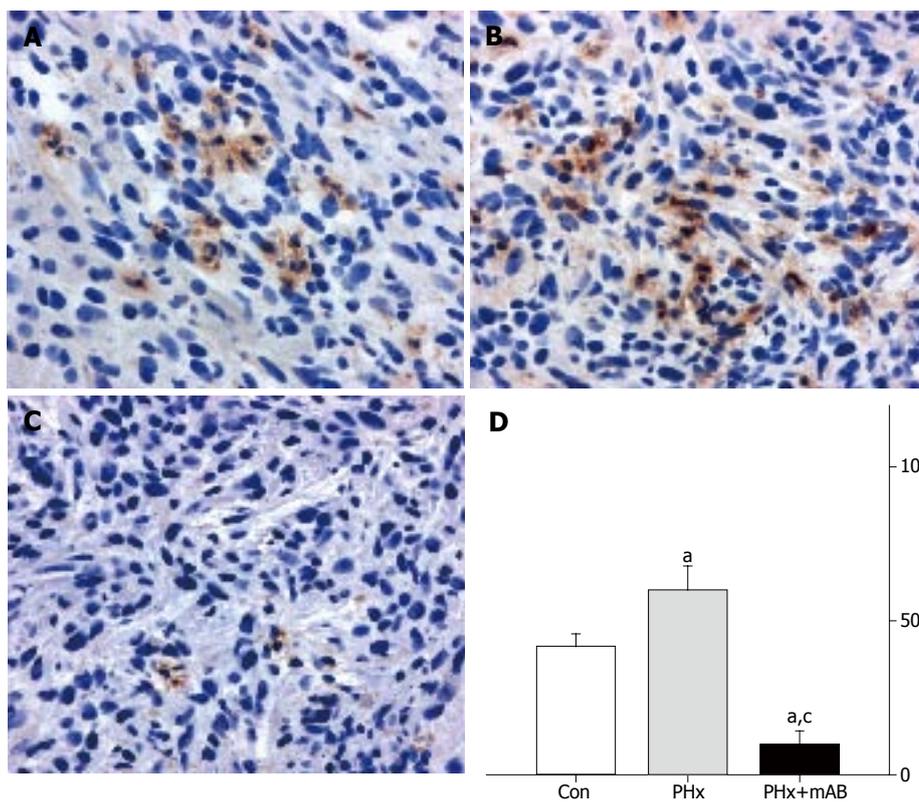


Figure 6 CXCR-2 immunohistochemistry and quantitative analysis of the number of receptor-positive cells (given in percent of all cells) revealed that within tumors of control animals (Con) ~40% of the cells express CXCR-2 (A and D). Hepatectomy (PHx) significantly increased CXCR-2 expression to ~60% (B and D). Of interest, additional blockade of MIP-2 (PHx+mAB) significantly reduced tumor cell CXCR-2 expression and inhibits significantly the liver resection-induced increase of CXCR-2 expression (C and D). Mean \pm SE; ^a $P < 0.05$ vs Con; ^c $P < 0.05$ vs PHx. Magnifications (A-C) $\times 175$.

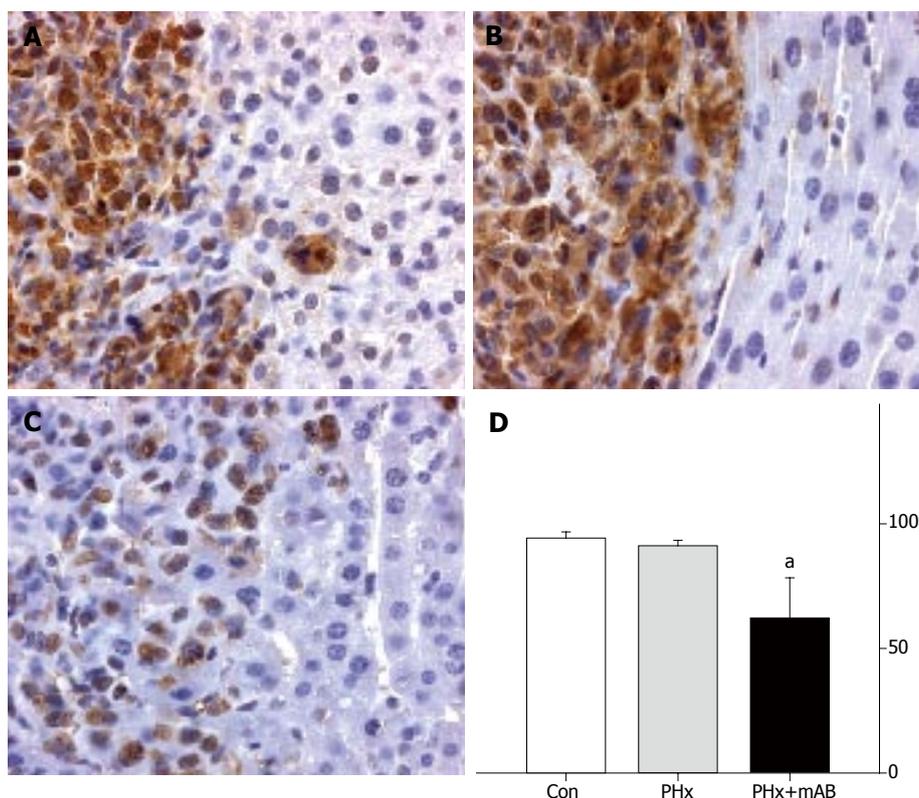


Figure 7 PCNA immunohistochemistry and quantitative analysis of the number of PCNA-positive cells (given in percent of all cells) in liver tumors of control mice (A, Con), after hepatectomy (B, PHx), and after hepatectomy and additional anti-MIP-2 treatment (C, PHx+mAB). Tumor cells display massive PCNA staining, in particular to those located within the tumor margin. By this, these positive cells sharply demarcate the tumor from the surrounding liver tissue (A, B, and C). Quantitative analysis revealed that neutralization of MIP-2 significantly reduces the number of PCNA-positive tumor cells (D). Mean \pm SE; ^a $P < 0.05$ vs Con. Magnifications (A-C) $\times 175$.

tumor vasculature is chaotically organized and does not follow a hierarchical branching pattern of normal vascular networks^[25]. As a result of this abnormal organization, the diameters of those tumor vessels are irregular, the blood flow is heterogeneous, and the endothelial lining is leaky^[25]. In the present study, the tumor microvasculature presented with a marked heterogeneity, as indicated by a relatively

high coefficient of variance of capillary and venular diameter within the angiogenic front. This is in line with the results of previous reports, which also demonstrated markedly irregular vessel diameters of tumors grown from other colon cancer cell lines at ectopic sites different from that of the liver^[26].

CXC chemokines with the three amino acids Glu-

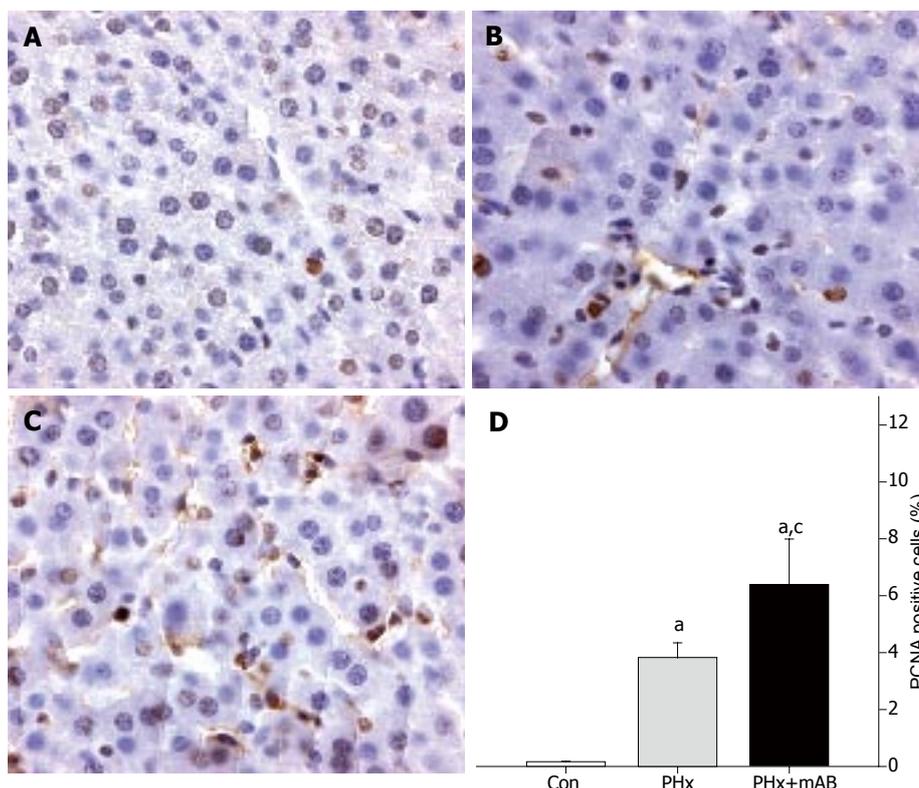


Figure 8 PCNA immunohistochemistry of normal liver tissue of control mice (A, Con), after hepatectomy (B, PHx), and after hepatectomy and additional anti-MIP-2 treatment (C, PHx+mAB). Quantitative analysis revealed that hepatectomy increases the number of PCNA-positive stained cells when compared to controls (D). Additional neutralization of MIP-2 further enhanced the number of PCNA-positive cells (D). Mean \pm SE; ^a $P < 0.05$ vs Con; ^c $P < 0.05$ vs PHx. Magnifications (A-C) $\times 175$.

Leu-Arg immediately amino-terminal to the CXC motif (ELR+) are thought to be angiogenic, while CXC (ELR-) chemokines are angiostatic^[27]. There is no information, however, on whether the CXC (ELR+) chemokine MIP-2 is involved in angiogenesis of tumors. In inflammation, Scapini *et al*^[28] demonstrated that MIP-2 induces angiogenesis *in vivo* and that this is mediated by the neutrophil-derived vascular endothelial growth factor-A. In addition, Keane *et al*^[29] showed that neutralization of MIP-2 by monoclonal antibodies inhibits the bleomycin-induced angiogenic activity in lung specimens. The present study provides evidence that MIP-2 is also involved in tumor angiogenesis, because the inhibition of the chemokine blunted the liver resection-induced acceleration of new blood vessel formation.

The mechanisms of inhibition of angiogenesis by blockade of MIP-2 are not clarified yet. Our intravital microscopic studies demonstrate that the hepatectomy-mediated increase in angiogenesis is associated with pronounced dilation of the newly formed microvessels. Although we have not studied the mechanism of this dilatory response, it may be speculated that it is caused by the action of VEGF, which is capable of inducing distinct vasodilation, in particular in ischemic tissue^[30]. VEGF is known as the predominant inducer of tumor angiogenesis and its promoter is preferentially activated within the angiogenic front of the tumor margin^[31]. This effect, however, cannot be attributed to the action of MIP-2, because anti-MIP-2 treatment could not abolish the liver-resection-induced dilation of the newly formed microvessels.

In the present study we found that inhibition of MIP-2 induced central cell death within the tumors. Previous studies on inflammation have demonstrated

that apoptotic cells are capable of enhancing secretion of MIP-2 by macrophages, triggering an inflammatory response to remove cell debris^[32,33]. Accordingly, the functional inhibition of MIP-2 in our experiments may have prevented the recruitment of inflammatory cells, and, thus avoided the removal of the cell debris from the center of the tumors.

In conclusion, we have demonstrated that MIP-2 significantly promotes liver resection-induced acceleration of angiogenesis and metastatic tumor growth. Our results therefore indicate that the neutralization of MIP-2 may be a potential strategy for additional anti-tumor therapy in patients undergoing liver resection.

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LIVER CANCER

Relationship between therapeutic efficacy of arterial infusion chemotherapy and expression of P-glycoprotein and p53 protein in advanced hepatocellular carcinoma

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Abstract

AIM: To investigate the relationship between the chemotherapeutic drug efficacy and the expression of P-glycoprotein (PGP) and p53 protein in advanced hepatocellular carcinoma (HCC).

METHODS: The study was conducted on 41 patients with advanced HCC who were treated by repeated arterial infusion chemotherapy. Biopsy specimens from the tumor were collected before the start of treatment in all the patients, and the specimens were stored frozen until immunohistochemical staining, which was performed after the start of treatment, to detect PGP and p53 protein expressions. Twenty of the forty-one patients were treated with an anthracycline drug (epirubicin hydrochloride; anthracycline group), and the remaining 21 were treated with a non-anthracycline drug (mitoxantrone hydrochloride in 11 patients and carboplatin in 10 patients; non-anthracycline group). The relationship between the chemotherapeutic efficacy and the results of immunostaining were compared between the two groups.

RESULTS: Before the start of the treatment, PGP-positive rate was 90.2% (strongly-positive, 36.6%) and p53 protein-positive rate was 34.1% (strongly-positive, 19.5%). In the anthracycline group, the response rate was 40.0%. The number of patients showing poor response to the treatment was significantly larger in the patients with strongly positive PGP expression ($P=0.005$), and their prognoses were poor ($P=0.001$). In the non-anthracycline group, the response rate was 42.9%,

and there was no significant relationship between the chemotherapeutic drug efficacy and the PGP or p53 protein expression. When only the data from the 11 patients treated with anthraquinone drug, mitoxantrone, were analyzed, however, the number of patients who showed poor response to treatment was significantly higher among the p53-positive patients ($P=0.012$), irrespective of the survival outcome.

CONCLUSION: The chemotherapeutic efficacy with an anthracycline drug for advanced HCC can be predicted by immunohistochemical analysis of PGP expression. Similarly, immunostaining to evaluate p53 protein may be useful to predict the response in patients treated with an anthraquinone drug.

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Key words: Arterial infusion chemotherapy; Hepatocellular carcinoma; P-glycoprotein; p53 protein

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INTRODUCTION

Transcatheter hepatic arterial embolization (TAE) is widely employed for the treatment of inoperable advanced hepatocellular carcinoma (HCC). However, TAE is unsuitable for the cases with a tumor embolus in the main trunk or the first branch of the portal vein, or for those with severe hepatic injury. For these cases, repeated arterial infusion chemotherapy (RAIC) with a reservoir may be useful, although it is still difficult, at present, to assert that RAIC would provide satisfactory therapeutic results.

Drug resistance is a serious problem in the treatment of HCC, and is mediated by various mechanisms, including those mediated by P-glycoprotein (PGP) and p53 protein expressions. PGP is known to act as an ATP-dependent pump that facilitates transport of substances,

including anticancer drugs, from within to outside the cells, facilitating the development of multidrug resistance, including that to anthracyclines^[1]. Moreover, resistance to apoptosis conferred by p53 protein expression is also thought to be a potential factor in the development of multidrug resistance in cancer cells^[2]. It has been reported that a mutant-type p53 protein may emerge in various types of cancers due to gene deletion, recombination, or point mutations. This mutant type of p53 protein has been reported to have a considerably longer half life as compared with its wild-type counterpart^[3], and it is thought to act as a dominant-negative inhibitor against the wild-type protein, influencing the anti-tumor effects of anticancer drugs^[4].

While PGP and p53 protein expressions have been reported in HCC, mainly from the examination of surgically resected specimens, only a few reports have discussed the relationship between the efficacy of chemotherapeutic agents and the expression of PGP in cases of HCC^[5,6]; the relationship between the outcome of arterial infusion chemotherapy and the expression of PGP and p53 protein remains particularly obscure. In this study, immunohistochemical analysis was conducted on frozen biopsy specimens obtained from patients with advanced HCC before the treatment to evaluate PGP and p53 protein expressions, and the therapeutic outcomes of RAIC in relation to the expression of these proteins were compared by the type of drugs that were administered.

MATERIALS AND METHODS

Patients

One hundred and thirty patients were diagnosed to have an unresectable advanced HCC and treated by RAIC at the Department of Medicine and Clinical Oncology, School of Medicine, Chiba University, and its affiliated institutes between April 1995 and June 1997. We could obtain tumor biopsy specimens before the initiation of RAIC from 41 of these patients, and stored these specimens at -80°C until the assay. Immunohistochemical staining for PGP and p53 expressions was performed on these specimens and the results were analyzed in relation to the therapeutic outcome in the patients.

Immunohistochemical staining for PGP and p53 protein

Immediately before the start of RAIC, two or three biopsy specimens were obtained percutaneously using a 21G biopsy needle under ultrasound guidance from 41 patients. The tissue specimens were embedded in OCT compound and immediately stored at -80°C.

For the immunohistochemical staining, frozen sections (5 µm thick) were prepared and fixed with acetone for 5 min. After pretreatment to prevent non-specific staining, the sections were allowed to react overnight at 4°C with mdr (Ab-1, diluted 1:250) (Oncogene Research Products, Cambridge, MA, USA) as the primary antibody for PGP. For p53 protein staining, the sections were allowed to react at room temperature for 1 h with DO-7 (diluted 1:40, DAKO, Denmark). The sections were then successively treated with the secondary antibody, and then stained by the avidin-biotinylated peroxidase complex method for

PGP, and the streptavidin-biotin method for p53 protein. Color development was conducted by DAB staining, and nuclear staining was conducted with Mayer's hematoxylin for PGP and methyl green for the p53 protein.

Light-microscopic examinations of the stained sections were conducted to detect positive staining for PGP on the cytoplasmic membranes or for p53 protein within the nuclei of the cells in the cancerous areas. At least 300 cells were examined, and the staining intensity was classified based on the percentage of positively stained cells as follows: negative (0-5%); weakly positive (6%-30%); and strongly positive (31% or more).

Arterial infusion chemotherapy

A 6F indwelling catheter (Anthon PU®; Toray, Tokyo) was introduced percutaneously through the left subaxillary artery (16 patients) or right femoral artery (25 patients) up to the level of the proper hepatic artery or common hepatic artery. The catheter was connected at the other end to a subcutaneously implanted reservoir (Port-A-Cath®, Pharmacia Deltec, St. Paul, MN, USA; or Cersite®, Toray, Tokyo)^[7,8].

An anticancer drug was administered over a period of 15 min once in every 4 wk at the outpatient clinic, after first confirming the patency of the catheter by fluoroscopy. Twenty, eleven, and ten of the forty-one patients received epirubicin hydrochloride, mitoxantrone hydrochloride, and carboplatin, respectively. The doses for each administration were set as follows: 70 mg/body surface area for epirubicin hydrochloride, 12 mg/body surface area for mitoxantrone hydrochloride, and 300 mg/body surface area for carboplatin. No other therapy was administered concomitantly in any of the patients. All the patients had underlying liver cirrhosis, and the diagnosis of HCC was confirmed histopathologically in all of them. Fifteen of the forty-one patients had received TAE prior to the RAIC with epirubicin hydrochloride at 40-70 mg/body surface area, while in the remaining 26 patients, RAIC was administered as the initial therapy. Informed consent was obtained from all the patients prior to tissue biopsy and RAIC. In this study, these patients were divided into anthracycline group (20 patients) treated with the anthracycline drugs, epirubicin hydrochloride and non-anthracycline group (remaining 21 patients) treated with the other drugs. No significant differences in the background characteristics were observed between the anthracycline group and the non-anthracycline group (Table 1). The therapeutic efficacies of the drugs were evaluated according to WHO guidelines. The anticancer drug administration was discontinued in the event of detection of any serious side effects, such as leucopenia, thrombocytopenia, or hepatic dysfunction. Observation of the patients was continued until September 30, 2003, by which date, all of the patients died.

Statistical analysis

The data were statistically analyzed using χ^2 test or Mann-Whitney's *U*-test. Differences at a *P* value of 5% or lower were considered to be statistically significant. The survival rate from the start of the RAIC was calculated by the Kaplan-Meier method and analyzed using the log rank test.

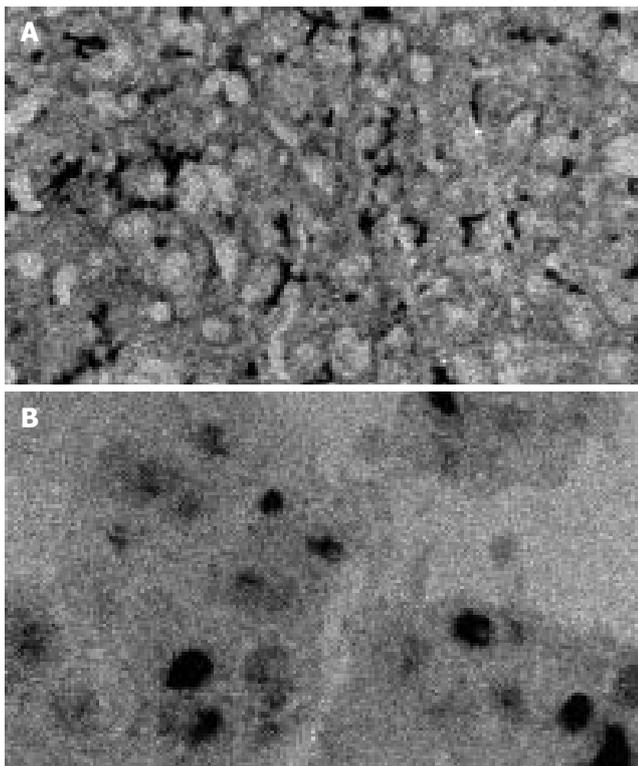


Figure 1 Immunohistochemical staining of PGP and p53 protein (×100). **A:** Strongly positive staining for PGP; **B:** Strongly positive staining for p53.

RESULTS

Immunohistochemical analysis for PGP and p53 protein expressions

Strongly-positive, weakly-positive, and negative PGP expressions were observed in 15 (36.6%), 22 (53.7%), and 4 patients (9.8%), respectively. Strongly-positive, weakly-positive, and negative p53 protein expressions were observed in 8 (19.5%), 6 (14.6%), and 27 patients (65.9%), respectively. Sections showing strong positivity for PGP and p53 protein expressions on immunohistochemical staining are shown in Figures 1A and B, respectively.

Of the 15 patients who had received TAE with anthracycline, 9 (60%) and 6 (40%) were found to be strongly and weakly positive for PGP, respectively, and none of the cases showed negative staining for PGP. Of the 26 patients who had not undergone TAE prior to the RAIC, 6 (23.1%), 16 (61.5%), and 4 (15.4%) patients were found to have strongly positive, weakly positive, and negative PGP expressions, respectively. Thus, the number of cases showing strong positivity for PGP expression was significantly higher among those who had a previous history of treatment with an anthracycline drug than those without such history ($P=0.036$). The percentage of cases showing p53 protein expression did not differ significantly among the patients with or without a history of treatment with an anthracycline drug.

No significant relationship was observed between the expression of PGP and that of the p53 protein (Table 2).

Relationship between therapeutic outcomes and immunohistochemical findings

Of the 41 patients, the 20 belonging to the anthracycline

Table 1 Patient characteristics

| | | Anthracycline group | Non-anthracycline group |
|----------------------------------|------------|---------------------|-------------------------|
| Age (yr) | Mean | 60.5±7.2 | 62.1±8.8 |
| Gender ¹ | Male | 18 | 19 |
| | Female | 2 | 2 |
| Tumor stage ^{1,2} | III | 1 | 1 |
| | IV | 19 | 20 |
| Child's class ¹ | A | 12 | 11 |
| | B | 6 | 9 |
| | C | 2 | 1 |
| Hepatitis virus ¹ | B(-) C(+) | 15 | 14 |
| | B(+) C(+) | 3 | 5 |
| | B(+) C(-) | 0 | 1 |
| | B(-) C(-) | 2 | 1 |
| Chemotherapy ^{1,3} | (-) | 13 | 13 |
| | (+) | 7 | 8 |
| HCC differentiation ¹ | Well | 1 | 2 |
| | Moderately | 14 | 15 |
| | Poorly | 5 | 4 |

Not significant (Mann-Whitney U-test). ¹Number of patients. ²According to the criteria for tumor stage according to TNM classification (UICC). ³Chemoembolization with epirubicin hydrochloride.

Table 2 Correlation between positivity of PGP and that of p53 protein

| Positivity | PGP positivity | | |
|------------------------------|-------------------|-----------------|----------|
| | Strongly-positive | Weakly-positive | Negative |
| p53 strongly-positive | 5 | 2 | 1 |
| p53 weakly-positive | 2 | 4 | 0 |
| p53 negative | 8 | 16 | 3 |
| Not significant (χ^2) | | | |

group received average arterial infusions of 11.4 ± 8.1 (range, 3-34) times during a mean follow-up period of 14.5 (range, 3-43) mo; 95%CI = 0.00-30.3. Of these 20 patients, 2, 6, 7, and 5 patients showed CR, PR, NC, and PD, respectively, yielding a response rate of 40.0% (95%CI = 18.5-61.5). In the 21 patients belonging to the non-anthracycline group, the mean number of arterial infusions and the mean duration of follow-up were 13.3 ± 9.5 (range, 3-38) times and 18.7 (range, 3-78) mo; 95%CI = 8.1-29.4, respectively. In this group, 3, 6, 8, and 4 patients showed CR, PR, NC, and PD, respectively, yielding a response rate of 42.9% (95%CI = 21.7-64.0).

The expression rate of PGP and p53 protein did not differ significantly between the anthracycline group and the non-anthracycline group (Table 3). Since the positive rate of PGP expression was very high in cases of HCC, the patients were further divided into those showing strongly positive expression (positive group) and those showing weakly positive or negative expression (non-positive group); a similar classification of the patients was conducted for p53 protein expression, into those showing strongly or weakly positive expression (positive group) and

Table 3 Correlation between PGP or p53 protein positivity and therapeutic efficacy by anticancer drugs

| Positivity | Anthracycline group (n = 20) | | | | Response rate (%) (95%CI) | Non-anthracycline group (n = 21) | | | | Response rate (%) (95%CI) |
|--------------------|------------------------------|----|----|----|-------------------------------|----------------------------------|----|----|----|---------------------------|
| | CR | PR | NC | PD | | CR | PR | NC | PD | |
| PGP ^{1,2} | | | | | | | | | | |
| Non-positive group | 2 | 6 | 3 | 0 | 72.7 (46.4-99.1) ^b | 3 | 5 | 5 | 2 | 53.3 (28.1-78.6) |
| Positive group | 0 | 0 | 4 | 5 | 0.0 (0.0-0.0) | 0 | 1 | 3 | 2 | 16.7 (-13.2-46.5) |
| p53 ^{1,3} | | | | | | | | | | |
| Non-positive group | 2 | 4 | 2 | 4 | 50.0 (21.7-78.3) | 3 | 6 | 6 | 2 | 52.9 (29.2-76.7) |
| Positive group | 0 | 2 | 5 | 1 | 25.0 (-5.0-55.0) | 0 | 0 | 2 | 2 | 0.0 (0.0-0.0) |

^b $P < 0.01$ vs positive group (χ^2). ¹Number of patients. ²Non-positive group: negative and weakly positive. Positive group: strongly positive. ³Non-positive group: negative. Positive group: weakly positive and strongly positive.

Table 4 Correlation between PGP or p53 protein positivity and cumulative survival rates

| Positivity | Number of patients | Survival rates (%) | | | Median survival time (mo) | 95%CI |
|-------------------------|--------------------|--------------------|-------|-------|---------------------------|------------------------|
| | | 12 mo | 24 mo | 36 mo | | |
| Anthracycline group | | | | | | |
| PGP | | | | | | |
| Non-positive | 11 | 90.9 | 54.6 | 9.1 | 26.5 | 15.8-37.2 ^b |
| Positive | 9 | 11.1 | 0.0 | 0.0 | 6.6 | 6.4-6.8 |
| P53 | | | | | | |
| Non-positive | 12 | 58.3 | 41.7 | 8.3 | 18.3 | 0.0-41.4 |
| Positive | 8 | 50.0 | 12.5 | 0.0 | 9.7 | 0.0-20.7 |
| Non-anthracycline group | | | | | | |
| PGP | | | | | | |
| Non-positive | 15 | 80.0 | 53.3 | 33.3 | 24.5 | 13.3-35.8 |
| Positive | 6 | 50.0 | 16.7 | 16.7 | 9.3 | 0.0-21.2 |
| P53 | | | | | | |
| Non-positive | 15 | 80.0 | 46.7 | 33.3 | 18.7 | 5.7-31.8 |
| Positive | 6 | 50.0 | 33.3 | 16.7 | 9.3 | 0.0-25.2 |

^b $P < 0.01$ vs positive group (log rank test).

those showing negative expression (non-positive group). As for the relationship of the therapeutic outcome to PGP expression in the anthracycline group, the number of non-responders was significantly higher in the positive group ($P = 0.005$); on the other hand, no such difference regarding PGP expression was found in the non-anthracycline group. In regard to the correlation of the therapeutic outcome with the expression of p53 protein, no significant differences between the positive and non-positive patients were found in either the anthracycline group or in the non-anthracycline group. But when only the data of the 11 mitoxantrone-treated patients of the non-anthracycline group were analyzed, 2 and 4 patients in the non-p53-positive group showed CR and PR, respectively, while 4 and 1 patients in the p53-positive group showed NC and PD, respectively, demonstrating the significant correlation between therapeutic outcome and the expression status of the p53 protein ($P = 0.012$). On the other hand, in the remaining 10 carboplatin-treated patients in this group, 1, 2, 4, and 2 patients in the non-p53-positive group showed CR, PR, NC, and PD, respectively, and the remaining one p53-positive patient showed PD, revealing the absence of any significant correlation between the therapeutic outcome and the p53

protein expression status.

The relationships between the positive rates for PGP or p53 protein and the survival rate are shown in Table 4. With regard to the PGP expression status in the anthracycline group, the survival rate was significantly lower in the positive group compared to non-positive group ($P = 0.001$); on the other hand, in the non-anthracycline group, no significant difference was observed between patients in the positive and non-positive group. As for the p53 protein expression status, no significant correlation was observed between the expression and the survival rate in either the anthracycline or the non-anthracycline group, including the 11 mitoxantrone-treated patients.

DISCUSSION

In conventional studies, the PGP-positive rate has been determined, mainly based on the examination of surgically resected specimens, to range from 52% to 92%^[5,9]. It has also been suggested that the expression of PGP decreases with decreasing degree of differentiation^[10]. In the present study, our patients had a relatively high PGP-positive rate of 90.2%, when weakly positive cases were

also included. It has been suggested that PGP expression might be induced by previously administered anthracycline chemotherapy in some cases^[11], and that following therapy, only the PGP-positive cells survived, resulting in the high overall positivity rate. However, further studies are required because the study population was small and we could not perform biopsies of the tumors for the duration of chemotherapy or after chemotherapy. On the other hand, the p53-positivity rate in patients with HCC has been reported to range from 12.5% to 51.2%^[6,10]. Consistent with these reports, the p53-positivity rate in our present study was 34.1%.

As to the relationship between the p53 tumor suppressor gene expression and the MDR1 gene expression, while some reported that the wild-type p53 protein represses the MDR1 promoter and the mutant-type p53 protein stimulates it, other investigators have suggested that the wild-type p53 stimulates the expression of this promoter^[12,13]. In our patients, no significant correlation was observed between the expression of PGP and that of the mutant-type p53 protein, suggesting that mutation of p53 may not regulate the expression of PGP, consistent with recently reported studies^[6,9,10]. In the present study, p53 protein was examined only by immunostaining using DO-7 which indicates the expressions of p53 protein variants. Therefore, a relationship between wild-type p53 protein and expressions of PGP could not be identified.

A relationship between PGP expression and the therapeutic efficacy of chemotherapeutic agents has been reported in cases of osteosarcoma and neuroblastoma^[14,15]. On the other hand, while no significant correlation was observed between the therapeutic outcome and the expression of PGP in 13 patients with HCC treated with hepatic arterial infusion of doxorubicin^[5], a significant correlation between the two was observed in 25 patients with advanced HCC treated by oral or intravenous administration of VP-16 or doxorubicin^[6]. In the present study on RAIC, the expression of PGP was significantly correlated with the therapeutic outcome and prognosis of the patients with advanced HCC, but only in the group treated with an anthracycline drug. These results, obtained from actually treated clinical cases of HCC, provide important evidence supporting the involvement of PGP in anthracycline resistance which was demonstrated in basic scientific studies^[1]. In addition, resistance mediated by alteration of nuclear topoisomerase, rather than PGP, has also been reported in patients receiving anthracycline drugs^[16], necessitating further studies on this issue. No significant correlation was found in this study between the expression of PGP and the therapeutic outcome or prognosis in patients treated with non-anthracycline drugs, such as an anthraquinone or a platinum-based drug. It is speculated that canalicular multispecific organic anion transporter (cMOAT) is involved in the excretion of cisplatin, and over-expression of cMOAT has been observed in an HCC cell line^[17].

As for the p53 protein, expression of a mutant-type of p53 protein has been reported to be associated with a poor prognosis^[10,18], and it has been suggested that apoptosis induced by anti-cancer drugs, such as doxorubicin, mitoxantrone, cisplatin and bleomycin, is

mediated, at least in part, by p53-dependent stimulation of the CD95 receptor/ligand system^[19,20]. However, no relationship between the chemotherapeutic drug efficacy and expression of the p53 protein has been reported until now in patients with HCC. In our present study, expression of p53 protein was not correlated with the chemotherapeutic drug efficacy or prognosis in patients treated with the anthracycline drug, suggesting a lesser importance of the expression of a mutant-type p53 protein, as compared with that of PGP, in the evaluation of the sensitivity of advanced HCC to anthracyclines. As for the anthraquinones among the non-anthracycline drugs, a fundamental study in a mitoxantrone-resistant cell line revealed that the expression of wild-type p53 protein induced cellular apoptosis^[21]; on the other hand, in relation to platinum-based drugs, expression of an abnormal type of p53 protein was reported to be associated with resistance to chemotherapy in cases of lung cancer^[22]. In our patients, treated with an anthraquinone, a significant correlation was observed between the expression of the p53 protein and the chemotherapeutic drug efficacy, but not the prognosis of patients with advanced HCC. On the other hand, no such correlation of the p53 expression was observed with either the chemotherapeutic drug efficacy or the prognosis in HCC patients treated with the platinum-based drug, carboplatin. Although further studies are required, these results indicate the possibility that evaluation of the expression of a mutant-type p53 protein may be useful for predicting the sensitivity of HCC to anthraquinone drugs.

In conclusion, our study demonstrates the existence of a correlation between the outcome of arterial infusion chemotherapy and the expression of PGP and p53 protein in patients with advanced HCC. Therefore, it is suggested that immunostaining for PGP may be useful for predicting the effectiveness of RAIC in cases treated with an anthracycline drug, and that p53 protein expression may be useful for predicting the outcome in cases treated with an anthraquinone drug.

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LIVER CANCER

Anti-hepatoma activity and mechanism of ursolic acid and its derivatives isolated from *Aralia decaisneana*

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Abstract

AIM: To investigate the anti-tumor activity of ursolic acid (UA) and its derivatives isolated from *Aralia decaisneana* on hepatocellular carcinoma both *in vitro* and *in vivo*.

METHODS: *In vivo* cytotoxicity was first screened by 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay. Morphological observation, DNA ladder, flow cytometry analysis, Western blot and real time PCR were employed to elucidate the cytotoxic mechanism of UA. Implanted mouse hepatoma H₂₂ was used to evaluate the growth inhibitory effect of UA *in vivo*.

RESULTS: UA could significantly inhibit the proliferation of HepG2 and its drug-resistance strain, R-HepG2 cells, but had no inhibitory effect on primarily cultured normal mouse hepatocytes whereas all the six derivatives of UA could not inhibit the growth of all tested cell lines. Further study on mechanism demonstrated that apoptosis and G₀/G₁ arrest were involved in the cytotoxicity and cleavage of poly-(ADP-ribose)-polymerase (PARP). Downregulation of cyclooxygenase-2 (COX-2) protein and upregulation of heat shock protein (HSP) 105 mRNA correlated to the apoptosis of HepG2 cells treated with UA. In addition, UA also could inhibit the growth of H₂₂ hepatoma *in vivo*.

CONCLUSION: UA is a promising anti-tumor agent, but further work needs to be done to improve its solubility.

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Key words: *Aralia decaisneana*; Ursolic acid; Hepatoma

INTRODUCTION

Hepatocellular carcinoma is one of the most common malignant neoplasms worldwide and one of the leading causes of malignancy-related death in China^[1,2]. Its therapy in clinic is still a big challenge.

Ursolic acid (UA) is a versatile compound, which possesses anti-cancer^[3,4], anti-inflammatory^[5,6], anti-HIV^[7] and immunomodulatory effects^[8]. There is a growing interest in the anti-tumor activities of UA. UA could act on almost all steps in the whole cancer process: initiation, promotion, progression, and metastasis. Multiple lines of evidence indicate that UA is a promising chemo-preventive agent both *in vivo* and *in vitro*. UA could inhibit Epstein-Barr virus activation induced by 12-*O*-tetradecanoylphorbol-13-acetate in Raji cells and two-stage mouse skin tumor promotion^[9,10]. The mechanism involved might associate with the inhibition of AP-1-mediated induction of COX-2 by disrupting PKC signal transduction pathway^[11]. UA could inhibit the proliferation of various cancerous cell lines by inhibiting DNA polymerase and topoisomerase^[12], inducing apoptosis through proteolytic activation of caspase-3 and/or other similar caspases^[5]. It was also reported that UA has anti-invasion and anti-metastasis activity by inhibiting nuclear factor-kappa B (NF- κ B) activation, down-regulating COX-2 and matrix metalloproteinase 9^[13].

Besides its cytotoxicity to hepatoma, it was reported that UA can exert its hepatoprotective action in mice and rats^[14-16]. The reason for the phenomenon is worth studying. Moreover, the growth inhibitory activity of UA on hepatocellular carcinoma *in vivo* is still unknown.

In the present study, UA and its six derivatives isolated from the bark of *Aralia decaisneana* were used to treat rheumatism, lumbago, hepatitis, nephritis, and diabetes mellitus in Chinese folk medicine. The cytotoxicity of these UA compounds to HepG2, its drug-resistance strain R-HepG2 and primarily cultured normal mouse and rat hepatocytes were examined in order to find their anti-hepatoma efficiency on both parental and drug-resistant hepatocellular carcinoma as well as their low toxicity.

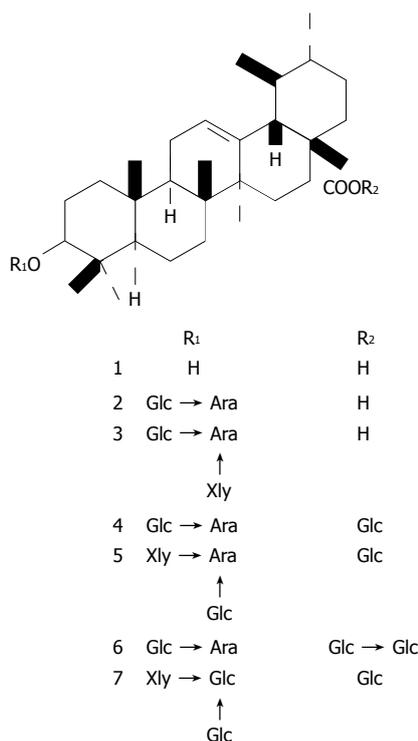


Figure 1 Structures of UA and its derivatives.

1. UA
2. UA-3-O-β-D-glucopyranosyl-(1→3)-α-L-arabinopyranoside,
3. UA-3-O-[[β-D-xylopyranosyl-(1→2)]]β-D-glucopyranosyl-(1→3)]-α-L-arabinopyranoside,
4. UA-3-O-β-D-glucopyranosyl-(1→3)-α-L-arabinopyranosyl-28-O-β-D-glucopyranoside,
5. UA-3-O-[[β-D-glucopyranosyl-(1→2)]]β-D-xylopyranosyl-(1→3)]-α-L-arabinopyranosyl-28-O-β-D-glucopyranoside,
6. UA-3-O-β-D-glucopyranosyl-(1→3)-α-L-arabinopyranosyl-28-O-β-D-glucopyranosyl-(1→6)-O-β-D-glucopyranoside,
7. UA-3-O-[[β-D-glucopyranosyl-(1→2)]]β-D-xylopyranosyl-(1→3)]-β-D-xylopyranosyl-28-O-β-D-glucopyranoside.

Morphological observation, DNA ladder, cell cycle analysis, Western blot and real time PCR were performed to elucidate the cytotoxic mechanism of UA on HepG2 cells. Mice bearing murine hepatoma H₂₂ were used to investigate the growth inhibitory effect of UA *in vivo*.

MATERIALS AND METHODS

Tissue culture and drug treatment

HepG2 (ATCC) cells were maintained in RPMI 1640 (Gibco) containing 10% FBS (Gibco), 2 mg/mL sodium bicarbonate, 100 μg/mL penicillin sodium salt and 100 μg/mL streptomycin sulfate. R-HepG2 (City University of Hong Kong) was maintained in the presence of 1.2 μmol/L doxorubicin (Sigma). Hepatocytes were isolated from normal Kunming mice (Experimental Animal Centre of Zhongshan Medical University) and SD rats (Experimental Animal Centre of Zoology, Chinese Academy of Sciences) with enzymatic perfusion technique as previously described^[17]. Logarithmically growing cells were used for all the experiments.

UA and its derivatives (Figure 1) were isolated from the bark of *Aralia decaisneana* by silica gel column chromatography and their structures were identified by spectral analysis^[18]. UA was dissolved in DMSO at the

concentration of 100 and 10 mmol/L in cellular and *in vivo* experiments, respectively and diluted in tissue culture medium and saline before use. Six UA derivatives were directly dissolved in tissue culture medium.

Cytotoxicity assay

HepG2 and R-HepG2 cells (1.5×10^4) as well as mouse and rat hepatocytes (8×10^3) were seeded in 96-well plates and treated with the compounds at various concentrations (3.125–100 μmol/L). Then the cells were incubated at 37 °C in an atmosphere containing 50 mL/L CO₂ for 48 h followed by MTT assay. IC₅₀ of the tested compounds on different cell lines were obtained from the concentration–effect curves.

Morphology observation

HepG2 cells were cultured in 3.5-cm dishes. UA was added to the medium at 20 μmol/L for 6, 12, and 24 h. A harringtonin-(20 μmol/L, 24 h) and vehicle-(0.1% DMSO) treated sample was regarded as a positive and negative control, respectively. After the treatment, all the cultures were incubated at 37 °C in an atmosphere containing 50 mL/L CO₂ for the indicated time. Photographs were taken under an inverted Leica fluorescence 40×10 microscope after acridine orange (AO)/ethidium bromide (EB) staining.

DNA ladder analysis

HepG2 cells were grown to 70–80% confluence and exposed to 20 μmol/L UA or harringtonin. Then the cultures were incubated at 37 °C in an atmosphere containing 50 mL/L CO₂ and collected at 0, 6, 12, and 24 h. Their genomic DNA was extracted and analyzed by gel electrophoresis^[19].

Flow cytometry analysis

Flow cytometry analysis was used to evaluate cell cycle distribution of HepG2 cells. HepG2 cells were treated with 20 μmol/L UA for 0, 6, 12, and 24 h, respectively, collected and fixed in 70% cold ethanol (–20 °C) overnight. After being washed twice with PBS, the cells were resuspended in PBS. RNA in the fixed cells was digested using RNase A (0.5 mg/mL) at 37 °C for 1 h. Finally, the cells were stained with 2.5 μg/mL propidium iodide (PI). The DNA content of the cells was then analyzed with an FACSCalibur instrument (Becton-Dickinson) at excitation 488/emission 600 nm. Data were analyzed by cell cycle distribution software (ModFit LT version 2.0, Verity Software House, USA).

Western blotting

After the treatment, cells were washed thrice with ice-cold PBS and collected with lysis buffer. Protein determination, SDS-PAGE and transfer were performed as previously described^[17]. Nitrocellulose membranes were then incubated with a monoclonal anti-PARP or polyclonal anti-COX-2 antiserum. Secondary antibody to IgG conjugated to horseradish peroxidase was used. The blots were probed with the ECL Western blot detection system according to the manufacturer's instructions.

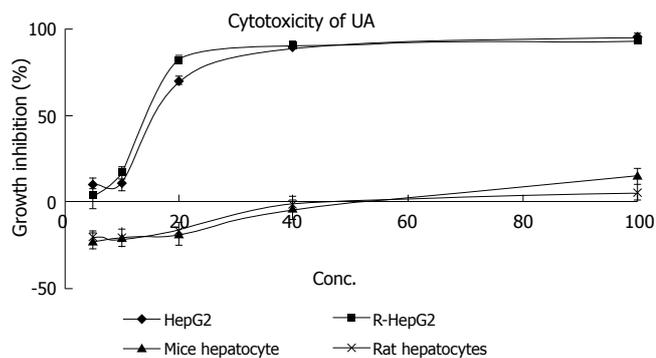


Figure 2 Cytotoxicity of UA to a panel of hepatocytes.

RT-PCR and real time PCR analysis

Total RNA was isolated from HepG2 cells using Trizol reagent according to the standard protocol and used to generate cDNA in each sample using the SuperScript II reverse transcriptase with oligo (dT) primers. Aliquots of total cDNA were diluted and added into each PCR reaction mixture together with 0.5 $\mu\text{mol/L}$ of forward and reverse primers of HSP 105 (F: CAGGATCCAGTTGTACGTGCTC; R: TGATGTGGAGGTTTCAGCACC, 197 bp) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, F: ATCAGCAATGCCTCCTGCA; R: CCTGCTTACCACCTTCTTGA, 355 bp) (Shanghai Biotech Engineering Service Limited Company). PCR amplification was performed on a GeneAmp 9700 PCR System (ABI). After denaturation of cDNA at 94 $^{\circ}\text{C}$ for 3 min, the cycling conditions were as follows: 32 cycles consisting of denaturation at 94 $^{\circ}\text{C}$ for 30 s, annealing at 59 $^{\circ}\text{C}$ for 30 s, and extension at 72 $^{\circ}\text{C}$ for 1 min. The PCR products were analyzed by agarose gel electrophoresis and Bio-Rad Chemi Doc. The mRNA expression levels were normalized to the expression of a housekeeping gene GAPDH. SYBR-Green-I fluorescence labeling method was used in real-time quantitative RT-PCR amplification on an ABI Prism 7000 SDS and analyzed with its software according to the standard protocol and manufacturer's instructions^[20]. The $2^{-\Delta\Delta CT}$ method was used in data analysis based on similar (<10%) amplification efficiency of the target and reference genes, which was achieved by the determination of ΔCT variations with template dilution^[21]. GAPDH was used as a reference gene for internal control.

Anti-tumor evaluation on implanted mouse H₂₂

Male CD-1 (ICR) mice (Beijing Vital Laboratory Animal Technology Company) weighing 20–22 g were used for the implantation of hepatoma H₂₂ (s.c.) which was maintained by weekly i.p. passages in CD-1 (ICR) mice, 0.2 mL ascites of 1:6 dilution from tumor-bearing mice 7 days after the tumor inoculation was implanted (s.c.) into the armpit region of the mice. Eight mice were treated i.p. with either UA or vehicle (1% DMSO in saline) once a day for 10 days 24 h after the tumor inoculation. Cyclophosphamide (10 mg/kg b.w.) was used as positive control. Tumor inhibition rate (TIR) was derived from $(1-T/C)\times 100$, where T is the mean tumor weight of the UA-treated group and C is the mean tumor weight of the negative control group.

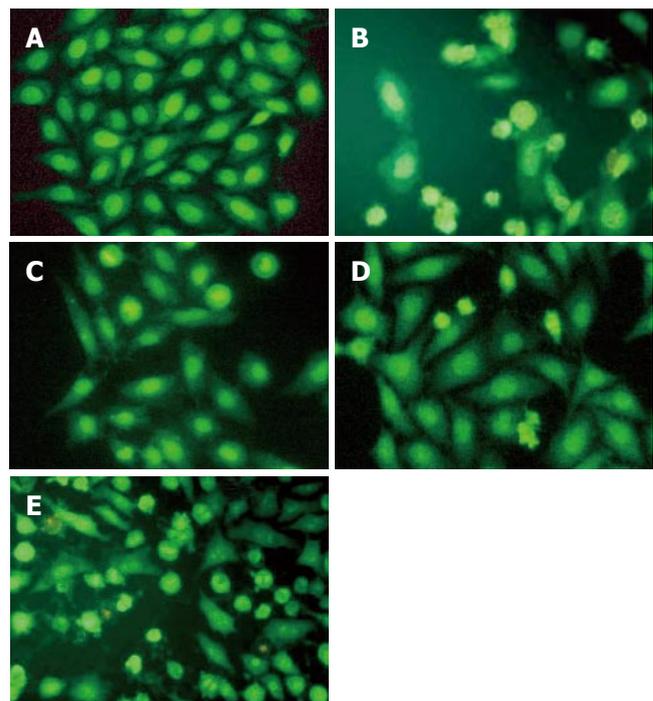


Figure 3 Morphological changes of HepG2 cells in response to harringtonin and UA. Cells were stained with AO/EB and observed under an inverted Leica fluorescence 40 \times 10 microscope. A: Normal HepG2 cells; B: harringtonin (20 $\mu\text{mol/L}$) for 24 h; C-E: UA (20 $\mu\text{mol/L}$) for 6, 12, and 24 h, respectively.

Statistical analysis

Student's t -test was used in all the experiments. $P < 0.05$ was considered statistically significant.

RESULTS

Selective cytotoxicity

Cytotoxicity of UA and its derivatives was determined by MTT assay. UA was demonstrated to have similar anti-proliferation activity on HepG2 and R-HepG2 cells in a concentration-dependent manner (Figure 2) with its IC_{50} value being 18 and 15 $\mu\text{mol/L}$, respectively, whereas it exhibited less growth inhibitory activity on primarily cultured normal mouse and rat hepatocytes and the IC_{50} value could not be detected at the tested concentration. On the contrary, six UA derivatives did not show any cytotoxicity to all the tested cells, while they possessed better solubility in water than UA.

Morphological changes of HepG2 cells

HepG2 cells treated with harringtonin and 20 $\mu\text{mol/L}$ UA showed similar and significant changes (Figure 3). Chromatin aggregation, nuclear, and cytoplasmic condensation only could be seen in a few cells after being treated with UA for 6 and 12 h. Partition of cytoplasm and nuclei into the membrane bound-vesicles (apoptotic bodies) was observed in the cells treated with harringtonin and UA for 24 h. At the same time, apoptotic cells were found at this time point.

DNA ladder analysis of HepG2 cells

DNA ladder was used to prove the apoptosis of HepG2 cells induced by UA. After treatment with 20 $\mu\text{mol/L}$

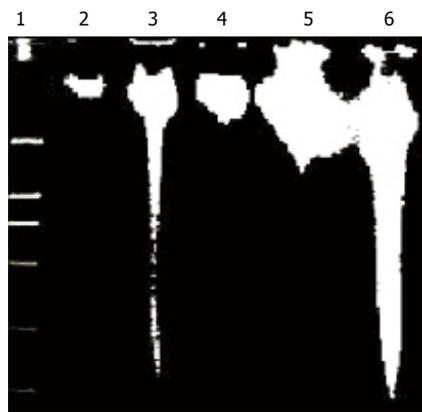


Figure 4 Induction of time-dependent fragmentation of nuclear DNA by harringtonin and UA. Genomic DNA of the treated cells was collected at different time points during the incubation. Lane 1: DNA marker (DL-2000); lane 3: harringtonin (20 $\mu\text{mol/L}$) for 24 h; lanes 2, 4–6: UA (20 $\mu\text{mol/L}$) for 0, 6, 12, and 24 h, respectively.

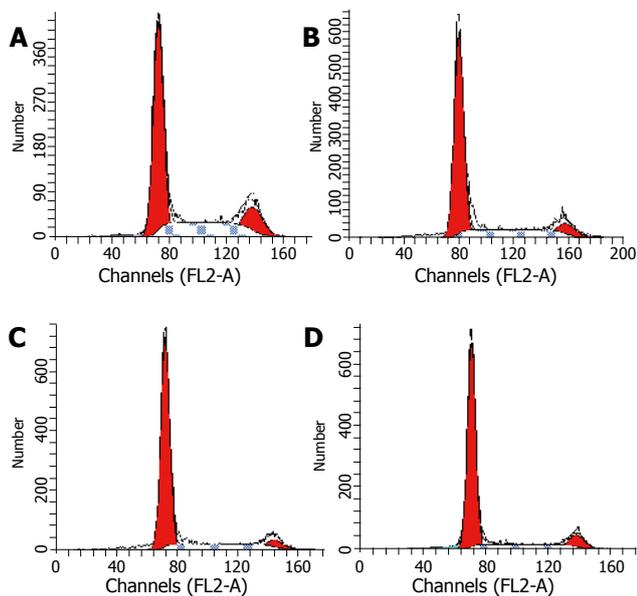


Figure 5 Cell cycle analysis of HepG2 cells treated with UA (20 $\mu\text{mol/L}$) for 0 (A), 6 (B), 12 (C), and 24 h (D), respectively. Cells were stained with PI and analyzed by flow cytometry.

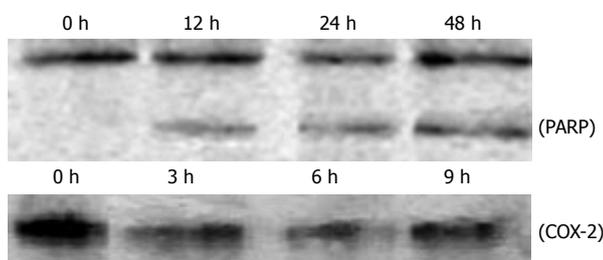


Figure 6 Cleavage of PARP and downregulation of COX-2 protein expression in HepG2 cells treated with UA. Cellular lysate protein (23 $\mu\text{g/lane}$) was loaded for 10% SDS-polyacrylamide gel electrophoresis, and subsequently transferred onto nitrocellulose. Immunoblots were probed with antibody specific for PARP and COX-2 protein. Lysates were from HepG2 cells treated with 20 $\mu\text{mol/L}$ UA for indicated times.

UA for different time points, evident DNA ladder was detected at 24 h on HepG2 cells treated with UA and

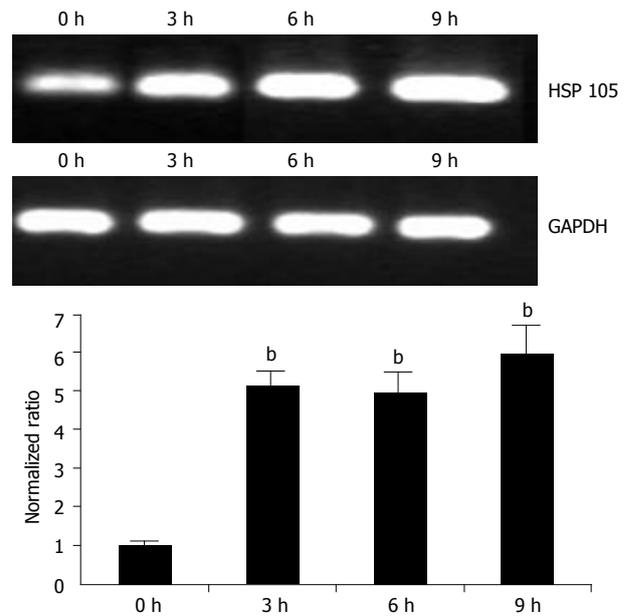


Figure 7 RT-PCR and real time PCR analysis of up-regulated HSP 105 gene in HepG2 cells in response to treatment with UA. HepG2 cells were treated with 20 $\mu\text{mol/L}$ UA for 0, 3, 6, and 9 h. Total RNA was isolated and subjected to semi-quantitative and quantitative RT-PCR analysis. GAPDH was used as a control. For quantitative analysis, data shown are mean \pm SD. $^{\text{b}}P < 0.001$ vs control, $n = 3$.

harringtonin (Figure 4), indicating the apoptosis observed in morphological inspection.

Cell cycle distributions in HepG2 cells

After being exposed to 20 $\mu\text{mol/L}$ UA for 0, 6, 12, and 24 h, respectively, the cell cycle progression of HepG2 cells showed evident changes (Figure 5). UA caused significant G_0/G_1 arrest with a concomitant decrease of cell population in S and G_2/M phases. The distribution of cell cycle in G_0/G_1 , S and G_2/M phases was 57.80%, 27.24%, and 14.96% at 0 h; 67.78%, 22.92%, and 9.3% at 6 h; 73.73%, 19.59%, and 6.69% at 12 h; 74.31%, 15.69%, and 10.0% at 24 h, respectively. When treated with UA for 12 and 24 h, higher percentage of cell cycle distribution in G_0/G_1 phase was detected than that at 6 h.

Cleavage of PARP protein and downregulation of COX-2

Exposure to 20 $\mu\text{mol/L}$ UA for 0, 12, 24, and 48 h, respectively, PARP was cleaved into 89- and 24-ku fragments, indicating that the apoptosis of HepG2 cells induced by UA was involved in caspase activation. After HepG2 cells were treated with UA for 0, 3, 6, and 9 h, respectively, COX-2 expression was significantly inhibited (Figure 6).

Upregulation of HSP 105 mRNA expression

HSP 105 belongs to HSP 70 super family and is closely related with apoptosis. The amplification efficiency of target and reference genes HSP 105 and GAPDH was quite similar (data not shown). The $2^{-\Delta\Delta C_t}$ method was used in data analysis. After being treated with 20 $\mu\text{mol/L}$ UA for 3, 6, and 9 h, respectively, HSP 105 mRNA expression was upregulated in a time-dependent manner both in RT-PCR and real time PCR (Figure 7).

Table 1 Tumor growth inhibition effect of UA in mice bearing H₂₂ (mean±SD, n = 8)

| Samples | Dosage (mg/kg) | Tumor weight (g) | Growth inhibition (%) |
|---------|----------------|------------------|-----------------------|
| Control | | 2.8±1.22 | |
| C.P. | 10 | 1.07±0.55b | 61.1 |
| UA | 30 | 1.27±0.90a | 54.6 |
| UA | 15 | 1.55±0.91a | 44.6 |

^aP<0.05, ^bP<0.01 vs control.

Tumor growth inhibitory effect

Twenty-four hours after tumor implantation, administration of UA (30 and 15 mg/kg b.w.) and cyclophosphamide (10 mg/kg b.w.) once a day for 10 days, could significantly suppress the growth of H₂₂ (Table 1). Significant body weight loss was observed in cyclophosphamide-treated group compared to the control group, whereas only slight body weight loss was observed in UA-treated group, suggesting that UA might possess anti-hepatoma effect *in vivo* with low toxicity.

DISCUSSION

In terms of cancer treatment, chemotherapy has serious limitations, namely the lack of selectivity of active ingredients and resistance of cancer cells to these chemicals^[22]. Thus, it is urgent to find new compounds that can kill both parental cancer cells and drug resistant cells and differentiate between normal and cancer cells in order to selectively kill cancerous cells with reduced toxicity. UA possesses good cytotoxicity on HepG2 and R-HepG2 cells, but no cytotoxicity to primarily cultured normal mouse and rat hepatocytes has been detected at corresponding concentrations. These results indicate that UA is a promising anti-tumor agent to parental and drug resistant HepG2 cells with low toxicity and could partially explain why UA inhibits the proliferation of hepatoma while possesses hepatoprotective activity. In addition, UA can lead to apoptosis, DNA ladder and PARP protein cleavage as well as G₀/G₁ cell-cycle arrest.

However, the cytotoxic mechanisms of HepG2 cells induced by UA are not completely understood. PARP is a substrate of apoptosis specific protease from the ICE-family (caspases). The 113 ku PARP is cleaved during apoptosis into 89- and 24-ku fragments which could serve as an early hallmark of apoptosis. In our study, cleaved PARP protein not only further proved apoptosis, but also it was implied that activation of caspases was involved in apoptosis of HepG2 cells treated with UA. COX-2 is an early-response gene that is highly inducible by mitogenic and inflammatory stimuli^[23-25]. Multiple lines of evidence suggest that COX-2 is important in carcinogenesis and overexpressed in cancer cells^[26,27]. NS-398, a representative of non-selective COX inhibitors can induce G₀/G₁ cell cycle arrest through inhibition of CDK2 and induction of p21 and p27^[28,29]. Upregulation of p21 could further stimulate mRNA transcription of cytochrome C and caspase 3 which induces apoptosis in the end^[30]. In the light of our study, UA could significantly inhibit COX-2 protein expression, suggesting that inhibition of

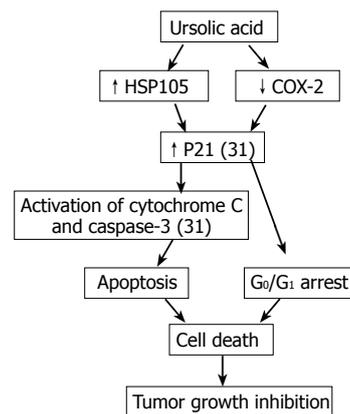


Figure 8 Summary of current understanding of cytotoxicity of UA to hepatoma cells. Functions with reference number represent known mechanism and bold words without reference number represent the mechanism revealed in our study.

COX-2 may correlate with increased p21 expression and subsequent activation of cytochrome C and caspase-3 in HepG2 cells treated with UA^[31].

HSP 105 belonging to heat shock protein (HSP) 70 super family acts as a chaperone and plays a role in control of cell proliferation and cellular aging. It was reported that overexpression of HSP105α could enhance stress-induced apoptosis, but not necrosis in mouse embryonal F9 cells^[32,33]. Some HSPs purified from murine tumors and used as vaccine are prophylactically and therapeutically effective in cancer immunotherapy models^[34]. Further more, MKT-077, a cationic rhodacyanine dye analog exerts its selective toxicity to cancer cells by binding to the HSP 70 family protein mot-2 and reactivating p53 function, thus resulting in increased p53 downstream gene p21 expression^[35]. Upregulation of HSP 105 and increase of p21 expression^[31] may contribute to apoptosis and G₀/G₁ cell-cycle arrest induced by UA, which are at least partially responsible for the selectivity of UA for cancer cells.

Tumor growth inhibitory effect in mice bearing hepatoma could predict the activity of corresponding tumors in human beings. Accordingly, the anti-neoplasm action of UA on mouse hepatoma H₂₂ indicates that it can be used to treat human hepatoma. However, few researches have been done on the pharmacokinetics of UA. We also did not monitor the concentration of UA in the blood of mice. Therefore, more attention should be paid to the active plasma concentration of UA in future.

In conclusion, UA is a potential anti-hepatoma agent with reduced toxicity both *in vitro* and *in vivo*. Summary of current understanding of anti-tumor activity of UA on hepatoma is shown in Figure 8. However, poor water solubility of UA has confined its use. Although its derivatives have good water solubility, these derivatives possess no anti-neoplasm activity. More work should be done to solve this problem.

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Role of CD80 in stimulating T lymphocyte activation

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Abstract

AIM: To observe biological characteristics of hepatocarcinoma cells before and after CD80 transfection and to compare the effect of CD80-transfected hepatocarcinoma cells on T lymphocyte activation.

METHODS: Retro virus vector carrying CD80 gene was transfected into HepG2 cells to establish CD80-transfected hepatocarcinoma cells (HepG2/hCD80). Flow cytometry (FCM) was performed to detect CD80 expression in the transfected cells. RT-PCR was used to evaluate CD80 expression at mRNA level. In the presence of anti-CD3 mAb, the proliferation of T lymphocyte was observed by MTT. Meanwhile, the expression of activated molecule marker CD25 was analyzed through FCM.

RESULTS: A stable cell line HepG2/hCD80 expressing the human CD80 was established. Growth curve showed that the molecule CD80 could obviously decrease the growth of tumor cells. HepG2/hCD80 was evidenced to have a potency to enhance T cell proliferation and up-regulate CD25 expression.

CONCLUSION: CD80 transfection can lower malignant phenotype of hepatocarcinoma cells. CD80 transfection has a down-regulatory effect to activated T cells *in vitro*.

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Key words: Hepatocarcinoma cells; CD80; Transfection; T lymphocyte activation

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INTRODUCTION

It has been widely accepted that T cell activation in the

course of anti-tumor immunity requires two distinct signals for T helper precursor cell expansion^[1,2]. Optimal activation of antigen-specific T cells requires not only the engagement of T-cell receptor (TCR) with Ag/major histocompatibility complex (MHC), but also co-stimulatory signals provided by antigen-presenting cells^[3,4]. Among the interactions of these co-stimulatory molecules, ligation of CD28/CTLA-4 and CD86 or CD80 is regarded as a major signal transduction pathway^[5]. As the first identified B7 family member, CD80 is widely expressed in a broad spectrum of tissues, even on some malignant tumor cells, thereby suggesting the potential mechanism of immune evasion of tumor cells^[6]. Cancer of the liver is regarded as a tumor of low immunogenicity. Although there is a high level of HLA I expression on the surface of hepatocarcinoma cells, it still has a low immunogenicity due to the absence or low expression of CD80^[7]. In this study, we established a CD80-transfected hepatocarcinoma cell line to observe the characteristic of HepG2/hCD80 and make further investigation on the role it plays in T cell activation.

MATERIALS AND METHODS

Materials

Retro virus vector pLNShB7 carrying hCD80 was presented by Professor Lu Daru of Fudan University. Package cell (singlephilic GP+E86, doublephilic GP+envAm12) and fibroblast cell NIH3T3 were kept by Jiangsu Hematological Research Institute and cultured in DMEM supplemented with 150 mL/L new born calf serum (NCS), and incubated at 37 °C in a humidified atmosphere containing 50 mL/L CO₂ in air. Hepatocarcinoma cell line HepG2 was purchased from Shanghai Zhongshang Hospital, cultured in RPMI 1640 medium supplemented with 150 mL/L NCS, and incubated at 37 °C in a humidified atmosphere containing 50 mL/L CO₂ in air. ELISA kit was purchased from Shanghai Senxiong Biological Co., Ltd. Mouse IgG-PE or IgG-FITC was purchased from Immunotech Company. MTT kit was purchased from Sigma Company. The mouse anti-human CD80 mAb was kindly gifted by Dr. Qiu Yuhua, Immunology Research Institute of Suzhou University.

Vector transduction and production of envAm12/CD80

Plasmid pLNShCD80 was transfected into *E. coli* JM109 and was confirmed by *EcoRI*. Purified vector DNA was transfected into GP+E86 cells using Lipofectamine. Singlephilic GP+E86/CD80 cells were selected by G418

(0.6 g/L) and then Ping-pong transfection strategy was adopted to produce doublephilic GP+envAm12/CD80. Supernatant of GP+envAm12/CD80 was collected and stored at -80°C .

CD80 virus transduction into HepG2 cells

HepG2 cells were incubated in Petri dish until they grew to 60% confluence. Then, the culture medium was replaced with 1:1 mixed virus supernatant and culture medium containing Polybrene at the final concentration of 4 mg/L. Such transduction process was repeated after a 3-h incubation at 37°C . After 24 h, the cells were digested and adjusted to proper concentration for selective culture in medium with G418 (0.6 g/L). The empty vector-transfected HepG2 (HepG2/mock) cells were prepared as a negative control in the following experiment.

Integration of CD80 gene

PCR was adopted to integrate NeoR gene in target cells and hCD80 gene. Primers for NeoR gene: 5'-CGTTG TCACT GAAGC GGGAA GG-3'. Primers for hCD80: 5'-ATTTT CTCT CTTT TGCCA GTAG-3'. Samples were initially denatured for 5 min at 95°C . Gene amplification was conducted for 32 cycles, each cycle consisted of denaturation at 94°C for 45 s, annealing at 58°C for 45 s, and polymerization at 72°C for 2 min, followed by an extra incubation at 72°C for 7 min to ensure full extension of the products. Products of amplification were analyzed on 12 g/L agarose gels in Tris-borate-EDTA, and stained with ethidium bromide.

Detection of CD80 expression by FCM

CD80-transfected HepG2 cells were cultured with anti-CD80 mAb (2 mg/L) at 4°C for 30 min. After being washed twice, the FITC-goat anti-mouse IgG as secondary antibody was added, followed by incubation at 4°C for 30 min. Then flow cytometry (FCM) was performed to detect the expression of CD80 after washes.

CD80 expression analysis by RT-PCR

Total RNA was isolated from 10^6 transfected cells with TRIzol reagents (Takara, Dalian, China) following the manufacturer's instructions. Using the total RNA as template, the first-strand cDNA of CD80 was obtained with oligo (dT) as a reverse primer following the manufacturer's protocol. Then the PCR amplification was carried out under the similar PCR conditions with the primers mentioned above.

Proliferation analysis of HepG2 and HepG2/hCD80 cells

The HepG2/hCD80 or HepG2/mock cells were seeded onto a 24-well plate with 1×10^5 /well. During the 8-day culture, cells' status was detected every day for an average value. According to the results, curves of cell growth were drawn and multiple proliferation time was calculated by the following formula: times of growth=maximum living cell number/living cell number of inoculation; multiple proliferation time=time from inoculation to the maximum cell number/time of growth.

Isolation of T lymphocytes

Two milliliters fresh peripheral blood was diluted with Hanks' balanced salt solution at a ratio of 1:3 and subjected to separation by 10 mL Ficoll density-gradient centrifugation at 2 000 r/min for 20 min. Then turbid fluid containing T cells was collected and subjected to another 10 mL Ficoll. After being washed twice with Hanks' balanced salt solution and separated by density-gradient centrifugation at 1 500 r/min for 15 min, the isolated T cells were cultured in RPMI 1640 at 37°C in a humidified atmosphere containing 50 mL/L CO_2 in air. Through the detection by FCM with PE-labeled anti-CD3 mAb, the T cell purification was about 70%.

Preparation of tumor vaccine

About 1×10^6 HepG2/CD80 or HepG2/mock cells were collected separately for Co-60 radiation (100 Gy) and then cultured in RPMI 1640 at 37°C in a humidified atmosphere containing 50 mL/L CO_2 in air for further usage.

Detection of proliferation index (PI)

HepG2/CD80 or HepG2/mock cells were seeded onto 96-well plates with 200 μL /well. Each was divided into two groups, and three double wells were set for each group. Lymphocytes were added into each well according to E:T ratio of 1:10. The mixture of lymphocytes and tumor cells was cultured in RPMI 1640 at 37°C in a humidified atmosphere containing 50 mL/L CO_2 in air. After a 3-day culture, MTT solution was added to each well to the final concentration of 10 g/L and the cells were further incubated for 5 h. Then 100 μL of isopropylalcohol was added to dissolve the crystal after the supernatant was removed by centrifugation at 3 000 r/min for 5 min and the wells were washed thrice with PBS. Then the absorbance of solution of each well was measured at 570 nm by a microplate reader (Bio-Rad). All the measurements were separately performed in triplicate. PI was calculated using the formula: $\text{PI} = (A_{570 \text{ nm of HepG2/hCD80}} - A_{570 \text{ nm of HepG2/mock well}}) / A_{570 \text{ nm of lymphocytes in each well}}$.

Analysis of surface antigen expression

HepG2/CD80 or HepG2/mock cells were seeded onto 24-well plates. Lymphocytes were added into each well according to E:T ratio of 1:10. The mixture of lymphocytes and tumor cells was cultured in RPMI 1640 at 37°C in a humidified atmosphere containing 50 mL/L CO_2 in air. After 2-, 5- and 7-day co-cultures, the lymphocytes, respectively, were stained with FITC-CD4 mAb, FITC-CD8 mAb, and FITC-CD25 mAb and incubated for 30 min, washed again with PBS. FCM was performed to detect the expressions of CD4, CD8, and CD25 on the surface of T cells.

Statistical analysis

The data was statistically analyzed by two-sided *t*-test, and $P \leq 0.01$ was considered statistically significant. Values were expressed as mean \pm SD.

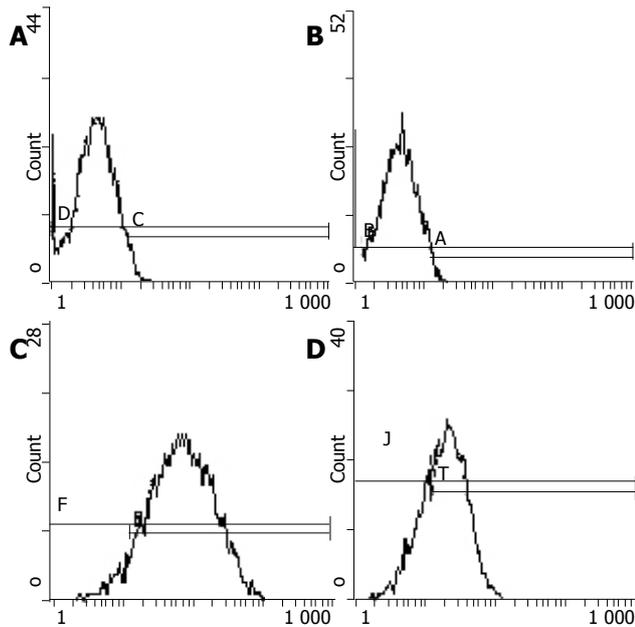


Figure 1 Flow cytometric analysis of CD80 expression in cells of different groups. **A:** HepG2/mock; **B:** negative control; **C:** HepG2/hCD80; **D:** positive control.

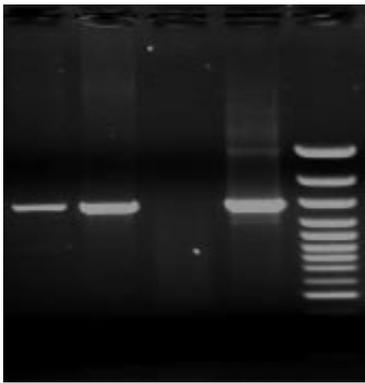


Figure 2 RT-PCR analysis of CD80 mRNA expression in different group cells. M: DL1000 molecule marker; lane 1: β -action; lane 2: positive control; lane 3: HepG2/mock; lane 4: HepG2/hCD80 cells.

RESULTS

Stable expression of CD80 in HepG2 cells

Retro virus vector pLNShB7 was transfected into HepG2 cells with the help of GP+envAm12 package cell. Drug resistant strains were selected by G418 to obtain doublephilic GP+envAm12/CD80 clones. FCM analysis showed that the expression of CD80 reached a rather high level (Figure 1). RT-PCR result further confirmed the expression of CD80 in the transfected cells at mRNA level (Figure 2).

Curve of cell growth and time of multiple proliferations

During 8 d of culture, the cells were counted each day for an average value. Curve of cell growth was drawn according to the results (Figure 3), which showed that the curve of HepG2/hCD80 cells was rising up very slowly, indicating that the growth ratio of HepG2/hCD80 cells was obviously lower compared to HepG2/mock cells. Multiple proliferation time of HepG2 cells was 13.86 h,

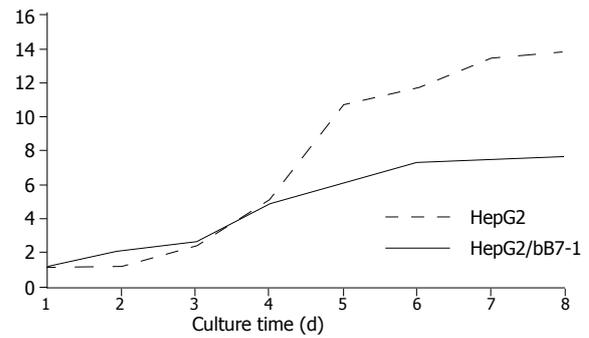


Figure 3 Curve of cell growth and time of multiple proliferation.

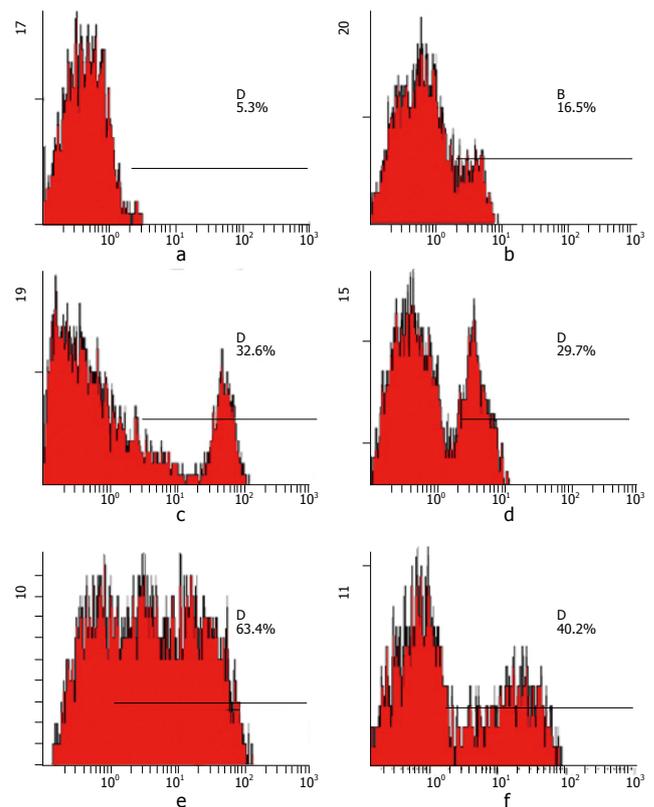


Figure 4 CD25 expression on T cells stimulated by HepG2/mock cells or HepG2/hCD80 cells. **A:** T cells stimulated by HepG2/hCD80 cells; **B:** T cells stimulated by HepG2/mock cells. Shaded histograms show reactivity with the isotype control. Each figure is representative of at least three experiments.

while that of HepG2/hCD80 was 25.09 h.

Surface molecular expression of T cells stimulated by HepG2/hCD80

After Co-60 radiation, HepG2/mock or HepG2/hCD80 cells were mixed with lymphocytes and cultured together. FCM was used to detect surface molecular expression in T cells on days 2, 5, and 7. The results showed that CD4,

CD8, and CD25 expressions were obviously higher in HepG2/hCD80 group than in HepG2/mock group on day 5 (Figure 4).

T cell proliferation stimulated by HepG2/hCD80

HepG2/CD80 or HepG2/mock cells were mixed with lymphocytes and cultured together as described above. After a 3-day co-culture, lymphocyte proliferation was examined by MTT. The results showed that PI of HepG2/hCD80 group (1.25 ± 0.12) was obviously higher than that of HepG2 group (0.43 ± 0.07 , $P < 0.01$).

DISCUSSION

Immune response of solid tumor is mainly cell immunity whose major effector cells are T lymphocytes. An optimal initiation of antigen-specific lymphocytes requires a combination of signals 1 and 2 (co-stimulatory signals). Hepatocarcinoma was regarded as a tumor of absent or low immunogenicity. Through testing different hepatocarcinoma cell lines, Tatsumi *et al.*^[8] discovered that only high level MHC-II molecules were expressed on the surface of hepatocarcinoma cells, while the expression levels of CD80 and CD86 turned to be very low. Although mRNA of both CD80 and CD86 was detected, the concentration was very low. Using FCM, we also examined CD80 expression level on the surface of HepG2 cells, which showed that only a few HepG2 cells expressed CD80 molecule with a percentage of only 3.66%. Obvious differentiation was found compared with positive control group. RT-PCR showed that human CD80 mRNA was about 605-bp in length (Figure 2), which was consistent with that of FCM results. Taken together the present and previous studies, we speculated that absence of CD80 expression contributed to the low immunogenicity of HepG2 cells.

CD80, a glycoprotein of 44/45 ku, is mainly expressed on activated B lymphocytes, macrophages and dendritic cells as well as tissues of chronic infection and tumor cells^[9]. Our experiment demonstrated that only a low level of CD80 expression was detected on the surface of HepG2 cells. Researches showed that some cytokines could upregulate CD80 expression on many kinds of cells. For example, IL-2 and IL-4 are able to enhance CD80 expression on B lymphocytes^[2]. But, generally speaking CD80 expression, even under above stimulation, is yet relatively low and unable to stimulate effective anti-tumor immune response. Therefore, by means of highly effective CD80 gene transfection to enhance B7 molecule expression at gene level, signal 2 was regained so as to effectively trigger anti-tumor immune response.

To effectively transfect CD80 gene, a vector of high efficiency and fewer side effects is necessary. Retro virus vector, at present, is a vector most widely used in the study of gene therapy. With this kind of vector, IL-2, p53 and other therapeutic genes could be transferred with high efficiency into hepatocarcinoma cells^[10]. So, we adopted retro virus as a carrier of CD80 gene. After selection by G418, FCM analysis showed that the positive ratio of HepG2/hCD80 group was about 92.2%, 28 times higher than the control group. Thus, a clone stably expressing

CD80 molecule was established, host cell HepG2 regained the ability of transducing the second signal, which is necessary to trigger T cell immune response.

By CD80 transfection, we not only established positive clones highly expressing CD80 molecules but also found that the growth curve of HepG2/hCD80 cells was rising up very slowly with the growth ratio obviously lower than that of HepG2 cells. Multiple proliferation time of HepG2/hCD80 was markedly longer than that of HepG2 ($P < 0.01$). So, CD80 transfection might change the status of tumor cells, while there is still a long way to go before clarifying its underlying molecular mechanisms.

T cell activation is crucial to immune response. After activation, a series of biochemical reactions occur, including signal transduction, gene activation and transcription, new molecule expression on cell surface, cytokine secretion and cells entering proliferation cycles^[11]. Following activation, interaction between IL-2 and IL-2R plays an important role in T cell proliferation^[12]. Absence of co-stimulatory signals might lead to T cell anergy, which could be explained by IL-2 decrease or no-response on IL-2 stimulation. On the surface of T cells, there are two IL-2 receptors, one is p55 (CD25) the other is p75 which has a relatively higher affinity, both have low affinity to IL-2^[13]. Resting T cells just express p75 and not p55, so it could only be activated by highly concentrated IL-2^[14]. A study showed that by the positive-feedback regulation of IL-2, CD25 could rapidly express and form complex with p75 to enhance the affinity of IL-2 and T cells^[15]. In this way, biological effects could be fully exerted with low level of IL-2 stimulation. After CD80 transfection, our experiment showed that CD25 expression on T cell surface was greatly improved and was obviously higher compared to control group, which may be explained by cascade effects of immune system^[16].

Enhancement of CD4 and CD8 expressions on T cell surface means the improvement of combined ability between TCR and MHC-I or MHC-II molecules^[17]. Compared with HepG2 group, HepG2/hCD80 group showed markedly increased CD4 and CD8 molecules and actively proliferated T cells, suggesting that the malignant status of hepatocarcinoma cells might be changed and enhancement of co-stimulatory signal could effectively stimulate T cell activation so as to exert strong anti-tumor effects.

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Characterization of H⁺ and HCO₃⁻ transporters in CFPAC-1 human pancreatic duct cells

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Abstract

AIM: To characterize H⁺ and HCO₃⁻ transporters in polarized CFPAC-1 human pancreatic duct cells, which were derived from a cystic fibrosis patient with the ΔF508 CFTR mutation.

METHODS: CFPAC-1 cells were seeded at high density onto permeable supports and grown to confluence. The cells were loaded with the pH-sensitive fluorescent dye BCECF, and mounted into a perfusion chamber, which allowed the simultaneous perfusion of the basolateral and apical membranes. Transmembrane base flux was calculated from the changes in intracellular pH and the buffering capacity of the cells.

RESULTS: Our results showed differential permeability to HCO₃⁻/CO₂ at the apical and basolateral membranes of CFPAC-1 cells. Na⁺/HCO₃⁻ co-transporters (NBCs) and Cl⁻/HCO₃⁻ exchangers (AEs) were present on the basolateral membrane, and Na⁺/H⁺ exchangers (NHEs) on both the apical and basolateral membranes of the cells. Basolateral HCO₃⁻ uptake was sensitive to variations of extracellular K⁺ concentration, the membrane permeable carbonic anhydrase (CA) inhibitors acetazolamide (100 μmol/L) and ethoxzolamide (100 μmol/L), and was partially inhibited by H₂-DIDS (600 μmol/L). The membrane-impermeable CA inhibitor 1-*N*-(4-sulfamoylphenylethyl)-2,4,6-trimethylpyridine perchlorate did not have any effect on HCO₃⁻ uptake.

The basolateral AE had a much higher activity than that in the apical membrane, whereas there was no such difference with the NHE under resting conditions. Also, 10 μmol/L forskolin did not significantly influence Cl⁻/HCO₃⁻ exchange on the apical and basolateral membranes. The administration of 250 μmol/L H₂-DIDS significantly inhibited the basolateral AE. Amiloride (300 μmol/L) completely inhibited NHEs on both membranes of the cells. RT-PCR revealed the expression of pNBC1, AE2, and NHE1 mRNA.

CONCLUSION: These data suggest that apart from the lack of CFTR and apical Cl⁻/HCO₃⁻ exchanger activity, CFPAC-1 cells express similar H⁺ and HCO₃⁻ transporters to those observed in native animal tissue.

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Key words: CFPAC-1; Pancreatic duct cells; HCO₃⁻; Intracellular pH

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INTRODUCTION

There are two HCO₃⁻ transport processes in the pancreatic ductal epithelium. The first is the secretion, mainly by the smaller ducts, of HCO₃⁻ rich isotonic fluid that serves to flush digestive enzymes down the ductal tree and to neutralize gastric acid entering the duodenum^[1,2]. The second HCO₃⁻ transport process is the flow-dependent exchange of luminal HCO₃⁻ for blood Cl⁻ that is generally considered to occur in the larger ducts, including the main duct^[1,2]. The physiological function of ductal HCO₃⁻ absorption (or salvage) may be protective as it will lower luminal HCO₃⁻ (and therefore luminal pH) during interdigestive periods when the flow is low or absent. A fall in luminal pH would reduce the activity of digestive enzymes within the static column of fluid in the ductal tree, thereby preventing damage to the ductal epithelium.

The initial step of HCO₃⁻ secretion is the accumulation of HCO₃⁻ within the duct cell^[1,2]. This can occur by two

mechanisms: (1) the forward transport of HCO_3^- by the $\text{Na}^+/\text{HCO}_3^-$ co-transporter (NBC); and (2) diffusion of CO_2 into the duct cell which is then hydrated to carbonic acid by carbonic anhydrase (CA), followed by the backward transport of protons via the Na^+/H^+ exchangers and H^+ -pumps. The HCO_3^- ions are then secreted across the apical membrane via $\text{Cl}^-/\text{HCO}_3^-$ exchangers (SLC26A3, DRA and SLC26A6, PAT1)^[3] and/or cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channels, which exhibit a finite permeability to HCO_3^- ^[4,5]. The exact mechanism how the SLC26 exchangers and apical Cl^- channels produce a high HCO_3^- secretion is controversial^[1,2]. Nevertheless, the key role of CFTR in HCO_3^- secretion is emphasized by the fact that the severity of the pancreatic phenotype in cystic fibrosis correlates best with the ability of mutant CFTRs to activate SLC26 exchangers, rather than their ability to conduct Cl^- ions^[6].

Rather less is known about the secondary, flow- and Cl^- -dependent absorption of HCO_3^- that occurs in the larger ducts. A luminal Na^+/H^+ exchanger (NHE3) and an electroneutral NBC (SLC4A7, NBCn1, NBC3) have been identified in the main duct of the mouse pancreas and probably explain HCO_3^- absorption across the apical membrane^[7-9]. There is a clear evidence that activity of these luminal HCO_3^- salvage transporters is downregulated following stimulation of HCO_3^- secretion by a rise in intracellular cyclic AMP^[7-9]. How the absorbed HCO_3^- exits the duct cell at the basolateral membrane is uncertain, but a candidate pathway is the basolateral $\text{Cl}^-/\text{HCO}_3^-$ exchanger (possibly AE2)^[10]. The AE2 must be switched off during stimulation as the prevailing ion gradients would favor Cl^- uptake and HCO_3^- efflux across the basolateral membrane.

Our current understanding of pancreatic ductal HCO_3^- secretion/salvage is mainly based on the experiments performed in animal tissues^[1]. Studies in human tissue have been largely confined to adenocarcinoma duct cell lines; however, with the exception of CAPAN-1, human duct cell lines do not express CFTR to any significant degree^[11]. An alternative approach is to use CFPAC-1 cells, which were derived from a 26-year-old cystic fibrosis (CF) patient with the ΔF508 mutation^[12]. The utility of CFPAC-1 cells is that they allow us to examine HCO_3^- transport not only in the diseased state, but also in the corrected state by transfecting the cells with wild-type CFTR. A number of previous studies have shown that wild-type CFPAC-1 cells express some of the key transporters involved in HCO_3^- secretion; e.g. the basolateral NBC^[13], anion exchanger-2 and the apical $\text{Cl}^-/\text{HCO}_3^-$ exchangers DRA and PAT-1^[14]. Moreover, the expression of apical $\text{Cl}^-/\text{HCO}_3^-$ exchangers was significantly increased in CFPAC-1 cells expressing functional CFTR, as was the stimulatory effect of ATP on luminal $\text{Cl}^-/\text{HCO}_3^-$ exchange^[15]. CFPAC-1 cells can also be reconstituted as polarized monolayers on permeable supports and the effects of various agents, such as angiotensin II^[16,17] and PKC^[18], have been studied using the short circuit current technique. Finally, in one study a stimulatory effect of ATP on HCO_3^- secretion from CFPAC cells was demonstrated by measuring the extracellular pH at the apical cell surface^[19].

A number of issues regarding the mechanism of pancreatic ductal HCO_3^- secretion remain controversial.

For instance, how much of the secreted HCO_3^- enters the lumen via CFTR and how much via SLC26 anion exchangers? How is coordinate regulation of apical and basolateral acid base transporters achieved during secretion and salvage? How do luminal signals regulate apical HCO_3^- and H^+ transporters? What is the molecular explanation for the strong outward rectification of HCO_3^- permeability exhibited by the apical membrane of the duct cell? A human pancreatic duct cell line, which reconstituted as a polarized monolayer allowing easy access to the luminal membrane, would be a very useful resource for answering these questions. An added bonus would be a model system that facilitates investigation of how CFTR expression affects duct cell transporters. Therefore, in this study, we aimed to establish whether polarized CFPAC-1 cells grown on permeable supports could fulfill these requirements. To do this, we investigated the HCO_3^- permeability characteristics of the basolateral and apical membranes of CFPAC-1 cells, and the spatial distribution of acid/base transporters.

MATERIALS AND METHODS

Materials and solutions

All laboratory chemicals were purchased from Sigma (Poole, Dorset, UK). 2',7'-Bis-(2-carboxyethyl)-5(6)-carboxyfluorescein-acetoxymethyl ester (BCECF-AM) and dihydro-4,4'-diisothiocyanostilbene-2,2'-disulfonic acid ($\text{H}_2\text{-DIDS}$) were from Molecular Probes Inc. (Eugene, OR, USA); 1-*N*-(4-sulfamoylphenylethyl)-2,4,6-trimethylpyridine perchlorate (STP) was kindly provided by Prof. C Supuran. Stock solutions of BCECF (2 mmol/L), acetazolamide (0.4 mol/L), ethoxzolamide (0.4 mol/L), amiloride (0.8 mol/L) and STP (10 mmol/L) were prepared in dimethyl sulfoxide (DMSO). Nigericin (10 mmol/L) and forskolin (50 mmol/L) were dissolved in ethanol and stored at -20°C . Polyester Transwells were supplied by Corning-Costar (Buckinghamshire, UK). The standard HEPES-buffered solution contained 130 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgCl_2 , 1 mmol/L CaCl_2 , 10 mmol/L Na-HEPES, and 10 mmol/L D-glucose (pH 7.4 with HCl). In the Na^+ -free HEPES solution, NaCl was replaced by 140 mmol/L *N*-methyl-D-glucamine (NMDG)-Cl and the Na-HEPES was replaced by equimolar HEPES-acid. The Cl^- -free HEPES solution contained 140 mmol/L Na-gluconate, 2.5 mmol/L K_2SO_4 , 6 mmol/L Ca-gluconate, 1 mmol/L Mg-gluconate, 10 mmol/L HEPES-acid, 10 mmol/L D-glucose (pH 7.4 with NaOH). The Na^+/Cl^- -free HEPES solution contained 130 mmol/L NMDG-gluconate, 2.5 mmol/L K_2SO_4 , 1 mmol/L MgSO_4 , 1 mmol/L CaSO_4 , 10 mmol/L HEPES-acid, 10 mmol/L D-glucose (pH 7.4 with gluconate). The standard HCO_3^- solution contained 115 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgCl_2 , 1 mmol/L CaCl_2 , 25 mmol/L Na- HCO_3 , 10 mmol/L D-glucose. In the K^+ -free HCO_3^- solution, KCl was replaced with equimolar NaCl. The high K^+ HCO_3^- solution contained the same ingredients as the standard HCO_3^- solution but the KCl and NaCl concentrations were 115 and 5 mmol/L, respectively. The Na^+ -free HCO_3^- solution contained NMDG-Cl instead of NaCl and choline HCO_3^- in place of NaHCO_3 . Cl^- -free

HCO_3^- solution contained 115 mmol/L sodium gluconate, 2.5 mmol/L K_2SO_4 , 6 mmol/L Ca-gluconate, 1 mmol/L Mg-gluconate, 25 mmol/L Na-HCO_3 , 10 mmol/L D-glucose. The Na^+/Cl^- -free HCO_3^- solution contained 115 mmol/L NMDG-gluconate, 2.5 mmol/L K_2SO_4 , 1 mmol/L MgSO_4 , 1 mmol/L CaSO_4 , 25 mmol/L choline- HCO_3^- , 10 mmol/L D-glucose (pH 7.4 with gluconate). 10 $\mu\text{mol/L}$ atropine was added to the solutions containing choline to prevent the possible activation of muscarinic receptors. In solutions containing NH_4^+ , the concentration of Na^+ or NMDG was replaced to maintain osmolality. All the solutions containing HCO_3^- were continuously equilibrated with 5% CO_2 -95% O_2 to maintain pH at 7.4.

Culture of CFPAC-1 cells

CFPAC-1 cells (passage number 27-60) were obtained from Prof. RA Frizzell (University of Pittsburgh, Pittsburgh, PA, USA) and were grown in Iscove's modified Dulbecco's medium supplemented with 100 mL/L fetal calf serum, 2 mmol/L glutamine, 100 units/mL penicillin and 0.1 mg/mL streptomycin and were cultured as described previously^[12]. The media was changed every 1-2 d. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 . Cell monolayers were prepared by seeding at high density (300-350 cells/ cm^2) onto polyester permeable supports (12 mm diameter, 0.4 μm pore size Transwells). Cell confluence was checked by microscopy and determination of transepithelial electrical resistance using an EVOM-G voltohmmeter (World Precision Instruments, Sarasota, FL, USA). Experiments were performed 4-6 d after seeding.

Measurement of intracellular pH

Intracellular pH_i was estimated with the pH-sensitive dye BCECF^[20,21]. CFPAC-1 cells were loaded with 2 $\mu\text{mol/L}$ BCECF-AM in both the apical and basal chambers in standard HEPES for 30-45 min at 37 °C. After loading, the Transwells were transferred to a perfusion chamber mounted on an inverted Nikon Diaphot microscope (Nikon UK, Kingston upon Thames, UK). Apical and basal bath volumes were 0.5 and 1 mL and the perfusion rates were 3 and 6 mL/min, respectively. All the experiments started and ended with standard HEPES solution perfusing both sides of the cells. Experiments were performed at 37 °C. pH_i was measured using a Life Sciences Microfluorimeter System (Life Sciences Resources, Cambridge, UK). About 10-15 cells were alternately excited with wavelengths of 440 and 490 nm, and the 490/440 fluorescence emission ratio was recorded at 535 nm over a sampling period of 256 ms. The resting pH_i of CFPAC-1 cells in standard HEPES solution was determined using the method described by Hegyi *et al.*^[21]. Calibration of 490/440 ratio to pH_i was obtained using the high- K^+ /nigericin method. Extracellular pH was stepped between 5.94 and 8.46.

Determination of buffering capacity

The intrinsic buffering capacity (β_i) of CFPAC-1 cells was estimated according to the NH_4^+ prepulse technique^[22]. Briefly, the cells were exposed to varying concentrations of NH_4Cl (0-30 mmol/L) while Na^+ and HCO_3^- were omitted

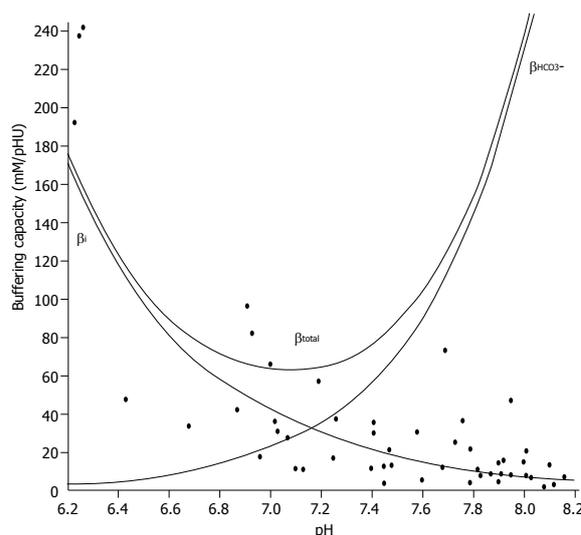


Figure 1 Buffering capacity of CFPAC-1 human pancreatic duct cells at different pH_i values. Polarized CFPAC-1 cells were exposed to varying concentrations of NH_4Cl (0-30 mmol/L), while Na^+ and HCO_3^- were omitted from the bath solution to block the Na^+ and HCO_3^- -dependent pH regulatory mechanisms. β_i was estimated by the Henderson-Hasselbalch equation ($n=50$). The total buffering capacity (β_{total}) was calculated as $\beta_{\text{total}}=\beta_i+\beta_{\text{HCO}_3^-}$, where $\beta_{\text{HCO}_3^-}$ is the buffering capacity of the $\text{HCO}_3^-/\text{CO}_2$ system. $\beta_{\text{HCO}_3^-}=2.3\times[\text{HCO}_3^-]_i$. $[\text{HCO}_3^-]_i$ is the intracellular concentration of HCO_3^- .

from the bath solution to block the Na^+ - and HCO_3^- -dependent pH regulatory mechanisms. β_i was estimated by the Henderson-Hasselbalch equation. The total buffering capacity (β_{total}) was calculated as $\beta_{\text{total}}=\beta_i+\beta_{\text{HCO}_3^-}$, where $\beta_{\text{HCO}_3^-}$ is the buffering capacity of the $\text{HCO}_3^-/\text{CO}_2$ system. $\beta_{\text{HCO}_3^-}=2.3\times[\text{HCO}_3^-]_i$. $[\text{HCO}_3^-]_i$ is the intracellular concentration of HCO_3^- .

Overall ΔpH_i , base flux

The overall ΔpH_i was measured by determining the pH_i immediately before and at the peak level during the administration of solutions containing $\text{HCO}_3^-/\text{CO}_2$ by averaging the values of 80 data points. The initial rates of ΔpH_i (over 30 s) were used to calculate transmembrane base flux $J(\text{B})$ using the equation $J(\text{B})=\Delta\text{pH}_i/\Delta t*\beta_i$. The β_i value used in the calculation of $J(\text{B})$ s was obtained from Figure 1 by using the average pH_i value over a 30-s period immediately before the administration of $\text{HCO}_3^-/\text{CO}_2$ (virtually no HCO_3^- in the cells). Therefore, we were most likely underestimating $J(\text{B})$ because of the increase in buffering capacity as HCO_3^- fluxes into the cell during the administration of the basolateral $\text{HCO}_3^-/\text{CO}_2$ solution. The only time we used β_{total} to calculate $J(\text{B})$ was when we administered basolateral standard $\text{HCO}_3^-/\text{CO}_2$ solution during perfusion with apical standard $\text{HCO}_3^-/\text{CO}_2$. We denote base influx as $J(\text{B})$ and base efflux as $-J(\text{B})$.

RT-PCR

Total RNA was extracted from CFPAC-1 cells grown in tissue culture flasks using RNAzol solution (Gibco-BRL) according to the manufacturer's instructions. For reverse transcriptase (RT)-PCR, 2 μg of RNA was reverse transcribed with 50 ng of random hexamer primers (GIBCO-BRL) in a final volume

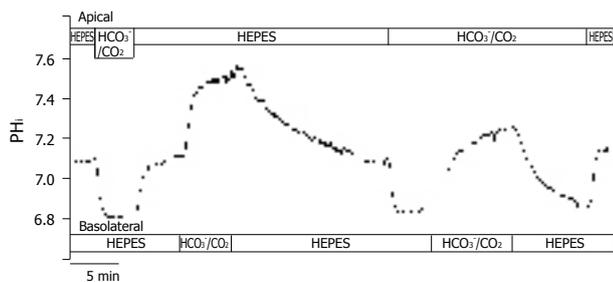


Figure 2 Functional polarities of CFPAC-1 cells. CFPAC-1 cells were grown to confluence on permeable supports, loaded with the pH-sensitive fluorescent dye BCECF-AM (2 $\mu\text{mol/L}$) and mounted into a perfusion chamber, which allowed the simultaneous perfusion of different solutions to the basolateral and apical membranes. The figure shows representative pH_i traces demonstrating cell polarity. The administration of standard $\text{HCO}_3^-/\text{CO}_2$ solution to the apical and the basolateral sides of the cells showed a marked difference in response ($n=12-20$).

of 20 μL using 50 U of Moloney murine leukemia virus RT (Fermentas) at 37 $^\circ\text{C}$ for 1 h, following the manufacturer's instructions. The primers used for PCR amplification were previously described^[23-25]. NHE1: 5'-CCAGCTCATTGCCTTCTACC-3' (sense) and 5'-TGTGTCTGTTGTAGGACCGC-3' (antisense) (length of amplified region 245 residues); AE2: 5'-GAAGATTCCTGAGAATGCCG-3' (sense) and 5'-GTCCATGTTGGCACTACTCG-3' (antisense) (length of amplified region 181 residues); pancreatic NBC1 (pNBC): 5'-ATGTGTGTGATGAAGAAGAAGTAGAAG-3' (sense) and 5'-GACCGAAGGTTGGATTTCTTG-3' (antisense) (length of amplified region 621 residues); glyceraldehyde-3-phosphate dehydrogenase (GAPDH): 5'-ATGGCACCGTCAAGGCTGAGA-3' (sense) and 5'-GCATGGACTGTGGTCATGAG-3' (antisense) (length of amplified region 371 residues). The PCR reaction was started with a 3-min 94 $^\circ\text{C}$ step and was followed by standard step-cycling conditions with 30 cycles of amplification utilizing *Taq* DNA polymerase (Fermentas); the reaction was ended by a 10-min 72 $^\circ\text{C}$ step. The cycling conditions for NHE1, AE2, and GAPDH were 94 $^\circ\text{C}$ for 30 s, 58 $^\circ\text{C}$ for 30 s and 72 $^\circ\text{C}$ for 30 s; and for pNBC1 were 94 $^\circ\text{C}$ for 30 s, 58 $^\circ\text{C}$ for 30 s and 68 $^\circ\text{C}$ for 3 min. RT-PCR products were separated by electrophoresis on a 20 g/L agarose gel containing ethidium bromide (0.5 $\mu\text{g/mL}$) and were visualized under ultraviolet light.

Statistical analyses

In agreement with Zsembery *et al*^[26], we noticed a variation in the rate and magnitude of HCO_3^- uptake between the different set of monolayers that we could not attribute to the passage number or time after seeding. To avoid errors arising from this fact, we (1) performed a particular experimental protocol on the same day using one set of cell cultures, (2) randomized the order in which monolayers in the same group were exposed to experimental maneuvers (i.e., exposure to inhibitors), and (3) always included internal controls where possible. Summary data in the figures are expressed as percent change from control and statistical analyses were performed using either Student's paired or unpaired *t* test or the analysis of variance as appropriate. *P* values <0.05 were considered statistically significant.

RESULTS

Transepithelial electrical resistance, resting pH_i and buffering capacity

The CFPAC-1 cells grown on polyester Transwells became confluent in 2-3 d as judged by visual observation. The net transepithelial resistance increased steadily over 4-5 d after seeding when it reached a plateau of $100 \pm 4 \Omega/\text{cm}^2$ ($n=88$). The resting pH_i of the cells in standard HEPES solution was 7.11 ± 0.08 ($n=6$).

Apical administration of $\text{HCO}_3^-/\text{CO}_2$

After perfusing both the apical and basolateral sides of the CFPAC-1 cells with standard HEPES solution, apical perfusion was switched to the standard $\text{HCO}_3^-/\text{CO}_2$ solution (Figure 2). We then observed a rapid acidification ($-17.5 \pm 1.5 \text{ mmol/L B/min}$, $n=20$) of pH_i , most likely caused by the diffusion of CO_2 into the cells. Following this, the pH_i reached a new approximate steady state value (6.82 ± 0.02 , $n=20$) and remained at this level until the standard HEPES solution was restored to the apical membrane. Restoration of standard HEPES solution induced a rapid alkalinization of pH_i towards the resting pH_i value probably brought about by the diffusion of CO_2 from the cells. These observations suggested that CO_2 influx was very fast across the apical membrane of CFPAC-1 cells, and that significant HCO_3^- influx did not occur.

Basolateral administration of $\text{HCO}_3^-/\text{CO}_2$

After perfusion of both the apical and basolateral membranes of CFPAC-1 cells with standard HEPES solution, the basolateral perfusion was switched to standard $\text{HCO}_3^-/\text{CO}_2$ (Figure 2). Upon changing the basolateral perfusate, there was a rapid alkalinization of pH_i ($\Delta\text{pH}_i = 0.48 \pm 0.03$, $J(\text{B}) = 15.0 \pm 2.3 \text{ mmol/L B/min}$, $n=12$), most likely induced by the transport of HCO_3^- into the cells. Removal of the standard $\text{HCO}_3^-/\text{CO}_2$ solution from the basolateral side induced a further small transient increase in pH_i consistent with efflux of CO_2 , followed by a slow acidification towards the resting pH_i value brought about by the extrusion of HCO_3^- (Figure 2). These observations suggested that HCO_3^- might cross the basolateral membrane of CFPAC-1 cells much faster than CO_2 . To rule out the possibility that basolateral HCO_3^- entry was at least partly due to CO_2 influx, followed by its rapid hydration to HCO_3^- ions, we perfused the apical side of CFPAC-1 cells with a standard $\text{HCO}_3^-/\text{CO}_2$ solution for 4 min (intracellular pCO_2 was increased) and then the same solution was added to the basolateral side (Figure 2). Under these conditions we still observed a rapid alkalinization of pH_i ($\Delta\text{pH}_i = 0.40 \pm 0.05$, $J(\text{B}) = 14.1 \pm 1.9 \text{ mmol/L B/min}$, $n=6$) which was not significantly different compared to the absence of apical $\text{HCO}_3^-/\text{CO}_2$.

Taken together with the effects of apical $\text{HCO}_3^-/\text{CO}_2$ administration, these data showed that CFPAC-1 monolayers exhibited functional polarity with respect to HCO_3^- transport. Subsequently, we used the apical administration of $\text{HCO}_3^-/\text{CO}_2$ to check the functional polarity of monolayers at the beginning of each experiment. The cells were considered to be polarized, if

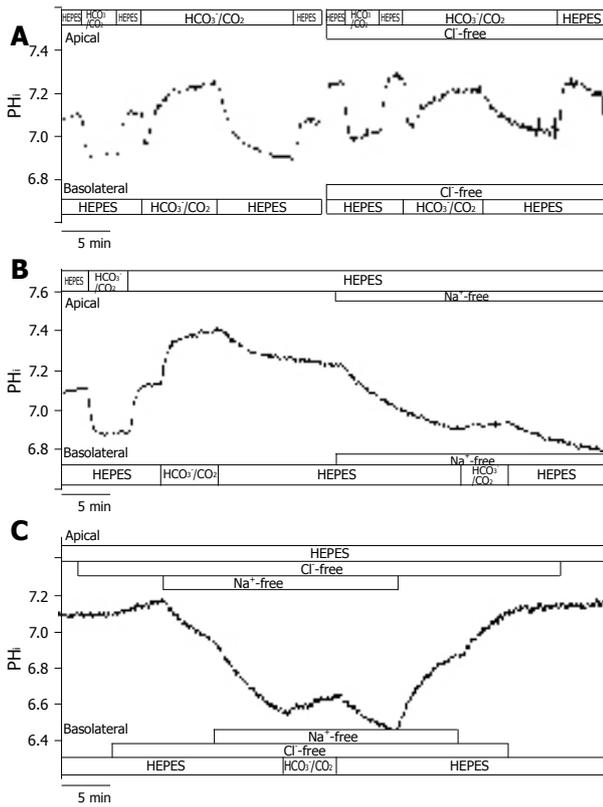


Figure 3 Basolateral HCO_3^- uptake – effects of Cl^- and/or Na^+ withdrawal. The figure shows representative pH_i traces. **A:** The rate of HCO_3^- uptake was significantly decreased in Cl^- -free conditions compared to standard conditions ($J(B) = 73.1 \pm 7.2\%$ and $100 \pm 8.5\%$, respectively, $n = 5$); **B:** The administration of basolateral $\text{HCO}_3^-/\text{CO}_2$ in Na^+ -free conditions ($n = 6$) greatly decreased ΔpH_i and $J(B)$ compared to standard conditions, suggesting that a large proportion of the HCO_3^- uptake was due to a Na^+ -sensitive process, most likely NBC; **C:** Interestingly, the decrease in the rate of HCO_3^- uptake was obviously ameliorated in the absence of both Na^+ and Cl^- ($n = 6$) compared to the Na^+ -free condition.

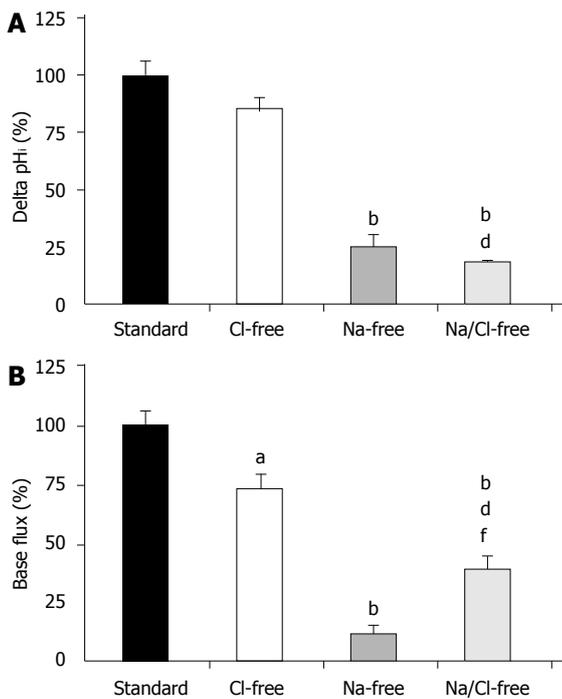


Figure 4 Basolateral HCO_3^- uptake – changes in (A) ΔpH_i and (B) $J(B)$ caused by the withdrawal of Cl^- and/or Na^+ . SE of the control groups (standard, black bar) is somewhat different for each of the respective ion-withdrawal groups. The SEs depicted on the standard bars are the maximum values observed. ^a $P < 0.05$ and ^b $P < 0.01$ vs control group ^d $P < 0.01$ vs Cl^- -free group and/or ^f $P < 0.01$ vs Na^+ -free group.

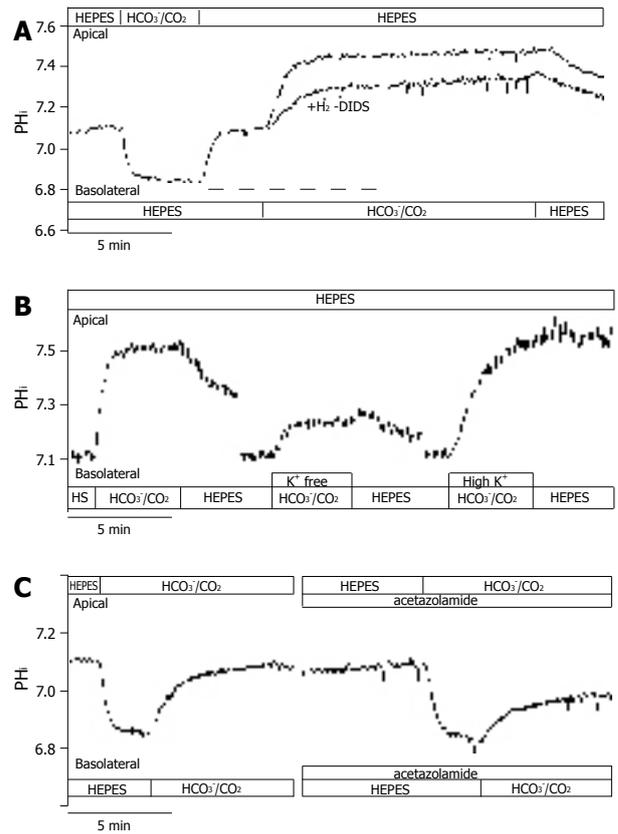


Figure 5 Basolateral HCO_3^- uptake – effects of $\text{H}_2\text{-DIDS}$, acetazolamide and varying extracellular K^+ concentration. The figure shows representative pH_i traces. **A:** The anion transport inhibitor $600 \mu\text{mol/L}$ $\text{H}_2\text{-DIDS}$ (added to the basolateral membrane of cells for 2 min before and during the administration of basolateral $\text{HCO}_3^-/\text{CO}_2$, dashed line, $n = 6$) significantly decreased the extent of alkalization (ΔpH_i) and rate of $J(B)$ ($n = 6$); **B:** Varying extracellular $[\text{K}^+]$ caused significant differences in ΔpH_i and $J(B)$ ($n = 9-11$); **C:** The application of the membrane-permeable carbonic anhydrase inhibitor acetazolamide ($100 \mu\text{mol/L}$, $n = 6$) significantly decreased the overall ΔpH_i and $J(B)$. HS: HEPES.

the pH_i remained constant after the initial decrease in pH_i caused by the diffusion of CO_2 into the cells.

Investigation of basolateral HCO_3^- uptake

In theory, the alkalization observed after the administration of basolateral standard $\text{HCO}_3^-/\text{CO}_2$ could be explained by the action of the NBC and the reversal of $\text{Cl}^-/\text{HCO}_3^-$ anion exchanger. The initial rate of HCO_3^- uptake was significantly decreased in Cl^- -free vs standard conditions ($J(B) = 73.1 \pm 7.2\%$ and $100 \pm 8.5\%$, respectively, $n = 5$, Figures 3A and 4); however, no significant difference was noted for the overall ΔpH_i for the two groups ($85.5 \pm 6.0\%$ and $100 \pm 6.3\%$, respectively). The administration of basolateral $\text{HCO}_3^-/\text{CO}_2$ greatly decreased ΔpH_i ($25.6 \pm 5.1\%$) and $J(B)$ ($11.7 \pm 2.3\%$) in Na^+ -free conditions ($n = 6$) compared to those ($100 \pm 7.5\%$ and, $100 \pm 10.7\%$, respectively) in standard conditions, suggesting that a large proportion of the HCO_3^- uptake was due to a Na^+ -sensitive process, most likely via an NBC (Figures 3B and 4). Interestingly, the decrease in the rate of HCO_3^- uptake was somewhat attenuated in the absence of both Na^+ and Cl^- [$n = 6$, $J(B)$ changed to $38.4 \pm 6.9\%$, Figures 3C and 4].

To further confirm the presence of NBC on the basolateral membrane of CFPAC-1 cells, we examined the recovery from a Na^+ -free acid load in the presence

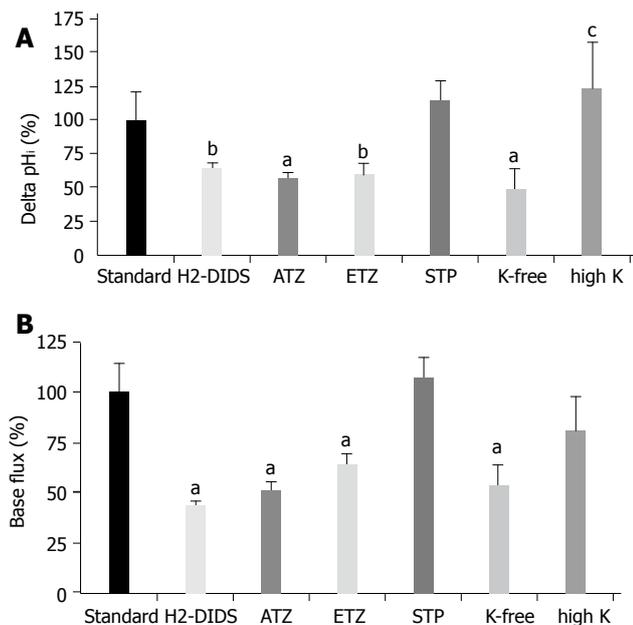


Figure 6 Basolateral HCO_3^- uptake – changes in (A) ΔpH_i (B) $J(\text{B})$ caused by application of H₂-DIDS, acetazolamide, ethoxzolamide, STP and varying extracellular K^+ concentration. Data are shown as mean \pm SE ($n=6-11$). SE of the control groups (standard, black bar) is somewhat different for each of the respective ion-withdrawal groups. The SE depicted on the control bar was the maximum values observed. ^a $P<0.05$ and ^b $P<0.01$ vs control group or ^c $P<0.05$ vs K^+ -free group. ATZ: acetazolamide; ETZ: ethoxzolamide; STP: 1-*N*-(4-sulfamoylphenylethyl)-2,4,6-trimethylpyridine perchlorate. The SE depicted on the standard bars was the maximum values observed.

of $\text{HCO}_3^-/\text{CO}_2$ by adding basolateral Na^+ with/without 300 $\mu\text{mol/L}$ amiloride (to block basolateral Na^+/H^+ exchange, $n=6$). After acid loading of cells, the basolateral addition of Na^+ stimulated a $J(\text{B})$ of 39.0 ± 4.4 mmol/L B/min (please see below the much lower values in HEPES solution). In contrast, the basolateral addition of Na^+ in the presence of amiloride decreased this value to 22.2 ± 3.6 mmol/L B/min, suggesting that a third pathway of acid transport is due to a basolateral NHE. The anion transport inhibitor H₂-DIDS (600 $\mu\text{mol/L}$, added to the basolateral membrane of cells for 2 min before and during the administration of basolateral $\text{HCO}_3^-/\text{CO}_2$, $n=6$), significantly decreased the extent of alkalization (ΔpH_i) to $63.2 \pm 4.7\%$ and rates of $J(\text{B})$ to $43.9 \pm 5.1\%$ ($n=6$, Figures 5A and 6). Varying the extracellular K^+ concentrations (K^+ -free, high K^+ , $n=9-11$) of the basolateral standard $\text{HCO}_3^-/\text{CO}_2$ solution induced significant differences between the ΔpH_i values vs the standard $\text{HCO}_3^-/\text{CO}_2$ solution ($41.5 \pm 24.5\%$, $139.6 \pm 15.0\%$ and $100 \pm 11.1\%$, respectively; Figures 5B and 6). The $J(\text{B})$ significantly decreased when K^+ -free $\text{HCO}_3^-/\text{CO}_2$ ($73.9 \pm 6.4\%$) was administered to the basolateral membrane, but was not affected by high K^+ , $\text{HCO}_3^-/\text{CO}_2$ ($96.6 \pm 11.0\%$) vs the standard $\text{HCO}_3^-/\text{CO}_2$ solution ($100.0 \pm 15.8\%$). Thus, our experiments showed that alteration of the membrane potential of CFPAC-1 cells, by varying basolateral extracellular K^+ concentration, modified the extent of HCO_3^- uptake. The application of the membrane permeable CA inhibitors acetazolamide (100 $\mu\text{mol/L}$, $n=6$, Figures 5C and 6) or ethoxzolamide (100 $\mu\text{mol/L}$,

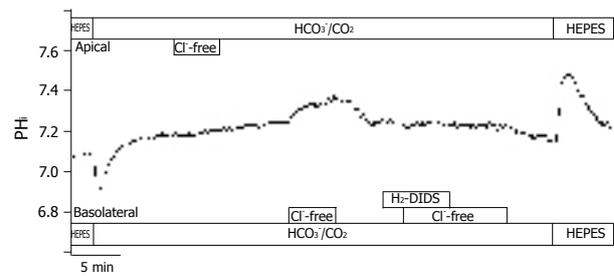


Figure 7 Localization of $\text{Cl}^-/\text{HCO}_3^-$ exchangers in CFPAC-1 cells. The figure shows representative pH_i traces ($n=6$). The localization of $\text{Cl}^-/\text{HCO}_3^-$ exchangers was performed by measuring the effect of changes in Cl^- gradient across the luminal or basolateral membranes on pH_i . In the presence of HCO_3^- , the removal of Cl^- from the apical membrane did not result in changes of pH_i . The removal of Cl^- from the basolateral membrane caused a ΔpH_i of 0.08 ± 0.01 and a $J(\text{B})$ of 4.37 ± 0.46 mmol/L B/min. Furthermore, the basolateral administration of 250 $\mu\text{mol/L}$ H₂-DIDS completely blocked pH_i changes caused by the withdrawal of Cl^- .

$n=6$) in the apical (standard $\text{HCO}_3^-/\text{CO}_2$ solution) and basolateral (standard HEPES solution) perfusions, 10 min before and during the administration of basolateral standard $\text{HCO}_3^-/\text{CO}_2$ solution, significantly decreased the overall ΔpH_i to $56.8 \pm 4.2\%$ or $59.0 \pm 9.8\%$, and $J(\text{B})$ to $50.5 \pm 4.6\%$ or $63.7 \pm 8.3\%$, respectively. The basolateral addition of the membrane impermeable CA inhibitor STP (10 $\mu\text{mol/L}$, $n=6$) in standard HEPES solution (the apical side of the cells was perfused with standard $\text{HCO}_3^-/\text{CO}_2$ solution), 5 min before and during the administration of basolateral standard $\text{HCO}_3^-/\text{CO}_2$ solution, did not significantly influence overall ΔpH_i ($114.3 \pm 14.3\%$) and $J(\text{B})$ ($106.7 \pm 17.9\%$, Figure 6).

Localization of $\text{Cl}^-/\text{HCO}_3^-$ exchangers in CFPAC-1 cells

The localization of $\text{Cl}^-/\text{HCO}_3^-$ exchangers was performed by measuring the effect of changes in Cl^- gradient across the luminal and/or basolateral membranes on pH_i . In the presence of HCO_3^- , the removal of Cl^- from the apical membrane did not result in changes of pH_i . The removal of Cl^- from the basolateral membrane caused a ΔpH_i of 0.08 ± 0.01 and a $J(\text{B})$ of 4.4 ± 0.5 mmol/L B/min ($n=6$, Figure 7). Furthermore, the basolateral administration of 250 $\mu\text{mol/L}$ H₂-DIDS (from 2 min before changing the solution to Cl^- -free $\text{HCO}_3^-/\text{CO}_2$) completely blocked pH_i changes caused by the withdrawal of Cl^- . However, 10 $\mu\text{mol/L}$ forskolin, 100 $\mu\text{mol/L}$ acetazolamide or 100 $\mu\text{mol/L}$ ethoxzolamide (from 10 min before changing the solution to Cl^- -free $\text{HCO}_3^-/\text{CO}_2$) did not significantly influence $\text{Cl}^-/\text{HCO}_3^-$ exchange on the apical and basolateral membranes ($n=6-8$, data not shown).

Localization of Na^+/H^+ exchangers in CFPAC-1 cells

In HEPES-buffered solutions, the removal of Na^+ from the perfusing solutions had a small acidifying effect on pH_i [$J(\text{B})=1.00 \pm 0.13$ mmol/L B/min, $n=12$]. After exposure and then removal of the NH_4^+ in the absence of Na^+ on both sides of the duct cells, pH_i reduced to 6.73 ± 0.03 ($n=12$), and stabilized at this level (Figure 8A). We could not detect any active H^+ -pumps in CFPAC-1 cells, since pH_i did not increase in the absence of extracellular Na^+ . Re-addition of Na^+ to the apical or the basolateral side caused pH_i recovery towards the resting values [$J(\text{B})=-6.6$

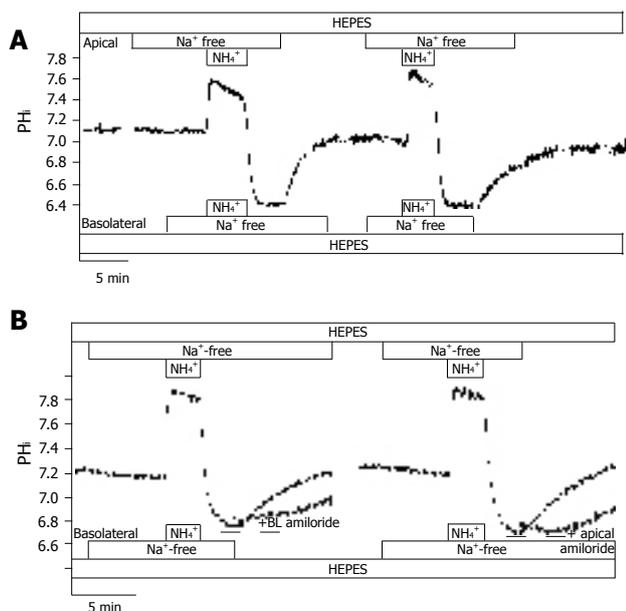


Figure 8 Localization of Na^+/H^+ exchangers in CFPAC-1 cells. The figure shows representative pH_i traces. **A:** After exposure to NH_4^+ in the absence of Na^+ on both sides of the duct cells, pH_i reduced to 6.73 ± 0.03 ($n=12$), and stabilized at this level. We could not detect any active H^+ -pumps in CFPAC-1 cells, since pH_i did not increase in the absence of extracellular Na^+ . Upon addition of Na^+ to the apical or the basolateral side prompted the pH_i to recover towards the resting values ($n=6-10$); **B:** The administration of 300 $\mu\text{mol/L}$ amiloride (dashed line) to the Na^+ -free (from 2 min before the addition of Na^+) and Na^+ -containing HEPES solutions prevented the recovery of pH_i in the presence of Na^+ ($n=6$). Removal of amiloride resulted in the recovery of pH_i to resting values.

± 2.6 mmol/L B/min and -7.5 ± 1.3 mmol/L B/min, respectively, $n=6-10$]. The administration of 300 $\mu\text{mol/L}$ amiloride to the Na^+ -free (from 2 min before the addition of Na^+) and Na^+ -containing HEPES solutions prevented the recovery of pH_i in the presence of Na^+ ($n=6$, Figure 8B). Removing amiloride resulted in the recovery of pH_i to resting values. Interestingly, the removal of apical Na^+ from the standard $\text{HCO}_3^-/\text{CO}_2$ solution perfusing CFPAC-1 cells caused a sudden but small acidification of pH_i ($\Delta\text{pH}_i = 0.14 \pm 0.01$ and $J(\text{B}) = -10.8 \pm 0.8$ mmol/L B/min, $n=6$), whereas we did not see any change in pH_i when we removed Na^+ from the basolateral side of the cells ($n=6$). Taken together, our experiments showed that there are Na^+/H^+ exchangers on both the apical and the basolateral membranes. Finding a Na^+/H^+ exchanger on the apical side was somewhat unexpected. A possible explanation for this would be that the cells were leaky to Na^+ ions. However, despite the great difference in Na^+ concentration (0 mmol/L *vs* 140 mmol/L) of the apical and basolateral perfusates, analysis of the Na^+ concentration of the solutions by mass spectrometry showed no contamination during perfusion.

Molecular identification of H^+ and HCO_3^- transporters

To further investigate the presence of different H^+ and HCO_3^- transporters in CFPAC-1 cells, we undertook RT-PCR analysis. We could detect mRNA expressions for pNBC1, AE2, and NHE1; the housekeeping gene GAPDH was used to normalize mRNA levels (Figure 9).

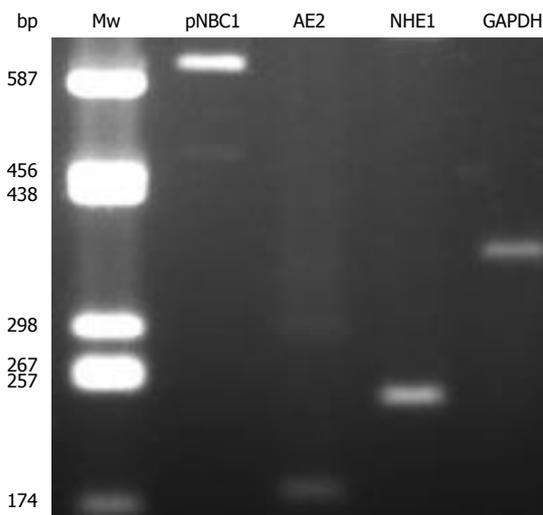


Figure 9 Expression of pNBC1, AE2, NHE1, and GAPDH mRNA in CFPAC-1 cells. Mw: molecular weight ladder (bp indicates number of base pairs).

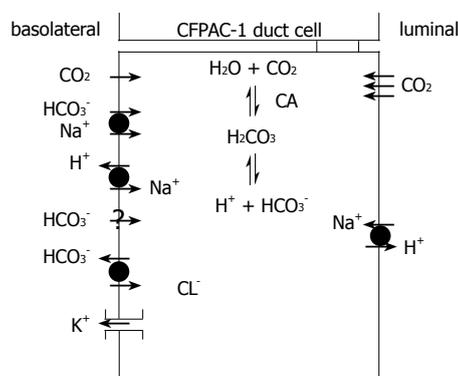


Figure 10 H^+ and HCO_3^- transport mechanisms in CFPAC-1 human pancreatic duct cells. The basolateral membrane expresses $\text{Na}^+/\text{HCO}_3^-$ co-transporters, Na^+/H^+ exchangers and $\text{Cl}^-/\text{HCO}_3^-$ exchangers plus an unidentified Na^+ -independent HCO_3^- entry pathway. These transporters facilitate the rapid flux of HCO_3^- ions into the cell. K^+ channels are also likely to be expressed on the basolateral membrane because altering extracellular K^+ concentration affects HCO_3^- influx. The apical membrane expresses Na^+/H^+ exchangers, but not CFTR and $\text{Cl}^-/\text{HCO}_3^-$ exchangers found in normal pancreatic duct cells. The apical membrane is an effective barrier to HCO_3^- influx from the lumen, but is freely permeable to CO_2 . CA: Carbonic anhydrase.

DISCUSSION

Our results showed the functional presence of the NBC and $\text{Cl}^-/\text{HCO}_3^-$ exchanger on the basolateral membrane, together with Na^+/H^+ exchangers on both the apical and basolateral membranes of CFPAC-1 cells (Figure 10). That there was no detectable $\text{Cl}^-/\text{HCO}_3^-$ exchange activity on the apical membrane was not a surprise as CFTR is known to be required for its activation^[3,27]. RT-PCR revealed the expression of pNBC1, AE2, and NHE1 mRNA.

CFPAC-1 cells clearly demonstrated a differential permeability to HCO_3^- at the apical and basolateral membranes. We found that the addition of 25 mmol/L $\text{HCO}_3^-/\text{CO}_2$ to the basolateral side of the cells caused a marked alkalization of pH_i , whereas exposing the apical membrane to the same solution caused a sustained acidification. These data suggest that the basolateral membrane of CFPAC-1 cells is much more permeable

to HCO_3^- than the apical membrane. Plasma membrane HCO_3^- and CO_2 permeabilities have been reported to vary in different species and tissues. In perfused rabbit gastric glands, the luminal membranes of parietal and chief cells were impermeable to HCO_3^- and CO_2 , while the basolateral membranes were permeable to both^[28]. Apical HCO_3^- permeability is relatively low in cultured bovine corneal endothelial cells^[29]. In contrast, HCO_3^- permeability of the apical side of intact colonic mucosa of guinea pig is markedly higher^[30]. Our results are in accordance with similar observations made by Ishiguro *et al.*^[31] using guinea pig ducts. Administration of 25 mmol/L $\text{HCO}_3^-/\text{CO}_2$ to the lumen of microperfused guinea pig pancreatic ducts decreased pH_i rapidly. Following this, no subsequent increase in pH_i was observed, indicating that little HCO_3^- was able to enter the cell from the lumen. HCO_3^- entry was detected only when the luminal HCO_3^- concentration was raised above 125 mmol/L. A low apical HCO_3^- permeability would make physiological sense in species, such as man and guinea pig, which can secrete near isotonic NaHCO_3^- . Our work is the first demonstration of $\text{HCO}_3^-/\text{CO}_2$ asymmetry in polarized human pancreatic duct cells. Finally, it seems that CFPAC-1 cells, which express ΔF508 CFTR, exhibit the same resistance to HCO_3^- influx across the apical membrane as native guinea pig duct cells. This suggests that the presence of functional CFTR in the apical membrane is not required for this membrane to act as an effective barrier to HCO_3^- influx from the lumen.

A large proportion of the basolateral HCO_3^- uptake was Na^+ -dependent and was partially inhibited by $\text{H}_2\text{-DIDS}$, therefore, it is most likely due to the action of pNBC1 . However, we also detected a novel Na^+ -independent HCO_3^- influx mechanism that was significantly increased in Cl^- -free conditions, the identity of which remains unknown. NBCs have been detected in the guinea pig^[32,33], rat^[34,35] and mouse^[36]. In contrast, Novak *et al.*^[37,38] could not detect any HCO_3^- uptake by NBC in freshly prepared rat pancreatic ducts using electrophysiological and pH_i measurements. Extracellular $\text{HCO}_3^-/\text{CO}_2$ did not increase the rate of pH_i recovery in experimental acidosis. The expressions of NBC mRNA and protein have been noted in the human pancreas^[39]. Immunofluorescence localized the transporter to the basolateral but not the apical membrane of the pancreatic duct cells. Interestingly, immunohistological studies have shown the NBC in both the apical and basolateral membranes of rat pancreatic duct cells^[35]. NBC1 mRNA was also detected in CFPAC-1 cells by Northern hybridization^[13]. In accordance with our findings, expression of NBC1 was also found in non-polarized cells by recovery from acidosis in the presence of HCO_3^- , Na^+ and amiloride and its sensitivity to the administration of 200 $\mu\text{mol/L}$ DIDS^[13]. cAMP had no effect on NBC activity in CFPAC-1 cells, whereas NBC activity was stimulated in CAPAN-1 cells expressing wild-type CFTR^[13]. The basolateral localization of the NBC was shown by $^{22}\text{Na}^+$ flux studies in CFPAC-1 cells stably transfected with wild-type CFTR grown on permeable supports (no data was reported for untransfected CFPAC-1 cells)^[13].

The application of the membrane permeable CA

inhibitor acetazolamide or ethoxzolamide partially inhibited the rate of basolateral base flux in CFPAC-1 cells, whereas the membrane-impermeable CA inhibitor STP^[40] had no such effect. Carbonic anhydrases are known to be coupled to HCO_3^- transporters, such as $\text{Cl}^-/\text{HCO}_3^-$ anion exchangers^[41-44], DRA ^[45] and NBC ^[46]. There are at least 14 different isoenzymes in the α -CA gene family present in vertebrates^[47]. CA I-III and VII are cytosolic, while CA IV, IX, XII and XIII are membrane-associated, V is mitochondrial and VI is secreted. Of the 14 different isoforms, CA II, IV, IX and XII were shown to be expressed in the human pancreas and cultured pancreatic tumor cells^[48]. Interestingly, CA II and IV expressions were found to be associated with apical HCO_3^- channels in human pancreatic duct cells CAPAN-1^[49], although the targeting of CA IV to the apical plasma membrane was impaired in the CFPAC-1 cells^[50]. Kivela *et al.*^[51] have clearly detected the transmembrane CA isoenzymes IX and XII on the basolateral membrane of the normal and pathological pancreas by immunohistochemistry. However, it is unlikely that the latter isoenzymes are associated with NBC as STP had no inhibitory effect on HCO_3^- uptake. Furthermore, in accordance with our results, Cheng *et al.*^[52] have shown in CAPAN-1 cells that HCO_3^- -dependent forskolin-induced I_{sc} could be significantly reduced by acetazolamide. Moreover, the CA inhibitor also had an inhibitory effect on dome formation, which was thought to be due to exchange of water and electrolytes^[49]. Taken together, intracellular CAs may be involved in the rapid basolateral transport of HCO_3^- by NBC. The enzyme converts HCO_3^- to carbonic acid and possibly maintains a concentration gradient for HCO_3^- across the basolateral membrane of CFPAC-1 cells.

Alteration of extracellular K^+ concentration significantly influenced HCO_3^- uptake in our experiments. The presence of K^+ channels in the basolateral membranes of CFPAC-1 cells is highly likely, and one report has identified ATP-sensitive K^+ channels^[53] in these cells. A basolateral K^+ conductance will influence the rate of HCO_3^- secretion by altering the cells membrane potential. In our experiments, high extracellular K^+ concentrations increased HCO_3^- entry, which was likely due to basolateral membrane depolarization, and a consequent increase in the electrochemical driving force for HCO_3^- entry on the electrogenic NBC. In contrast, low extracellular K^+ concentration inhibited HCO_3^- uptake, probably due to membrane hyperpolarization.

Novak and Greger^[37] demonstrated that the basolateral membrane of rat intra- and inter-lobular pancreatic ducts had a relatively large K^+ conductance, which could be decreased by the K^+ channel blocker Ba^{2+} . The luminal membrane had no significant K^+ conductance. Interestingly, Fong *et al.*^[54] detected K^+ conductances not only on the basolateral but also on the apical sides of HPAF human pancreatic duct cells.

CFPAC-1 cells exhibited Na^+/H^+ exchange activity on both the apical and basolateral membranes (the latter is most likely to be due to NHE1). Na^+/H^+ exchangers were identified in guinea pig^[10,28-30], rat^[55,56] and mouse^[8] pancreatic ducts. These exchangers were localized to the basolateral membrane of intra/interlobular duct cells.

However, the main and common segments of the rat pancreatic ducts also showed Na^+/H^+ exchange activity in the apical membrane^[10]. There is also evidence for an apical Na^+/H^+ exchanger in bovine main pancreatic duct segments^[57]. Furthermore, the main pancreatic duct of mice expressed NHE1 in the basolateral membrane, and NHE2 and NHE3 in the luminal membrane^[8]. Disruption of the NHE2 gene had no effect on luminal H^+ transport, while disruption of the NHE3 gene reduced luminal Na^+ -dependent H^+ efflux by 45%. The remaining 55% of the luminal Na^+ -dependent H^+ efflux from NHE3-knockout mice was inhibited by 50 $\mu\text{mol/L}$ HOE694. Na^+ -dependent H^+ transport mechanisms were inhibited by cAMP, therefore, they are thought to be inhibited during stimulated secretion and are most likely to be active during the resting state^[8]. CFTR in the pancreatic duct not only affected the activity, but also the expression of NHE3 protein^[7]. NHE3 expression was reduced by about 50%, luminal Na^+ -dependent and HOE694-sensitive recovery from acid load was reduced by 60% in the ducts of $\Delta 508$ CFTR mice^[7]. To our knowledge, we are the first to detect NHEs on both the apical and basolateral membranes of human pancreatic duct cells. The presence of NHE on the apical membrane was unexpected, since it acted against HCO_3^- secretion. We do not know which section of the pancreatic duct CFPAC-1 cells originate from, but they might be from the main region where the expression of the apical NHEs was noted in animal studies. In accordance with our results, failure of pHi to recover from acid loading in the absence of extracellular Na^+ suggests that there is little H^+ -pump activity in the unstimulated guinea pig ducts^[32]. This is in contrast with the observations made in rat^[10] and pig^[58] pancreatic ducts, which showed H^+ -pump activity.

The role of apical $\text{Cl}^-/\text{HCO}_3^-$ exchangers in pancreatic ductal HCO_3^- secretion is a controversial issue. One hypothesis is that all of the HCO_3^- is secreted on an anion exchanger and that CFTR's role is to activate the exchangers and to provide a route for the recycling of Cl^- across the apical membrane^[59]. An alternative proposal is that HCO_3^- secretion occurs on the exchanger until the luminal concentration reaches about 70 mmol/L after which HCO_3^- exits the duct cell via CFTR^[60]. Previously, it has been shown that CFPAC-1 cells express AE2 on their basolateral membrane (which was also confirmed in our study) and SLC26A3 (DRA) and SLC26A6 (PAT1) on their apical membrane, and that expression of the apical SLC26 exchangers is upregulated by CFTR^[14]. In accordance with these data, we observed basolateral $\text{Cl}^-/\text{HCO}_3^-$ exchange activity (which was inhibited by $\text{H}_2\text{-DIDS}$) in wild-type CFPAC-1 cells, but no apical activity, presumably due to the lack of CFTR. We also found that forskolin had no effect on either the basolateral or apical anion exchange in CFPAC-1 cells.

In conclusion, apart from the lack of CFTR and apical $\text{Cl}^-/\text{HCO}_3^-$ exchange activity, CFPAC-1 cells have similar H^+ and HCO_3^- transporters to those observed in native animal tissue. Moreover, intracellular CAs appear to play a role in the basolateral uptake of HCO_3^- . Taken together, polarized CFPAC-1 human pancreatic duct cells are likely to be a useful model to study H^+ and HCO_3^- transporters,

and their dependence on CFTR, in human pancreatic duct cells.

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BASIC RESEARCH

Inhibitory effects of interferons on pancreatic stellate cell activation

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be tested regarding their *in vitro* efficiency. Growth inhibition by IFN- γ action requires STAT1.

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Abstract

AIM: To analyze and to compare the effects of interferon (IFN)- α , IFN- β , and IFN- γ on pancreatic stellate cell (PSC) activation *in vitro* and to elucidate the molecular basis of IFN action.

METHODS: PSCs were isolated from rat's pancreatic tissue, cultured and stimulated with recombinant rat IFNs. Cell proliferation and collagen synthesis were assessed by measuring the incorporation of 5-bromo-2'-deoxyuridine (BrdU) into DNA and [3 H]-proline into acetic acid-soluble proteins, respectively. Apoptotic cells were determined by FACS analysis (sub-G₁ peak method). Exhibition of the myofibroblastic PSC phenotype was monitored by immunoblot analysis of α -smooth muscle actin (α -SMA) expression. To assess the activation of signal transducer and activator of transcription (STAT), Western blots using phospho-STAT-specific antibodies were performed. In studies on STAT1 function, expression of the protein was inhibited by siRNA.

RESULTS: IFN- β and IFN- γ , but not IFN- α significantly diminished PSC proliferation and collagen synthesis. IFN- γ was the only IFN that clearly inhibited α -SMA expression. Under the experimental conditions used, no enhanced rate of apoptotic cell death was observed in response to any IFN treatment. IFN- β and IFN- γ induced a strong increase of STAT1 and STAT3 tyrosine phosphorylation, while the effect of IFN- α was much weaker. Inhibition of STAT1 expression with siRNA was associated with a significantly reduced growth-inhibitory effect of IFN- γ .

CONCLUSION: IFN- β and particularly IFN- γ display inhibitory effects on PSC activation *in vitro* and should

INTRODUCTION

Pancreatic stellate cells (PSCs) are the main source of extracellular matrix (ECM) proteins in pancreatic fibrosis^[1-3], a common feature of chronic pancreatitis and pancreatic cancer^[4,5]. They are closely related with hepatic stellate cells (HSCs), the key effector cells in liver fibrosis^[6]. In response to pro-fibrogenic cytokines and ethanol metabolites, PSCs exhibit a myofibroblastic phenotype that is characterized by the expression of α -smooth muscle actin (α -SMA), proliferative activity, a loss of the typical retinoid-containing fat droplets, and enhanced ECM synthesis^[3,7]. Similar phenotypic changes, considered as PSC activation, are induced when isolated PSCs are plated on cell-culture dishes^[1,2,8-10]. The molecular principles of PSC activation *in vivo* and *in vitro* have been extensively analyzed in the past few years. Studies indicate that platelet-derived growth factor (PDGF) exerts strong mitogenic effects on PSCs, while transforming growth factor-beta (TGF- β) has been suggested as the most potent stimulator of ECM synthesis^[11,12]. Furthermore, intracellular signal transduction pathways mediating PSC activation have, in part, been deciphered. Thus, we and others have recently shown that mitogen-activated protein kinases are key mediators of activation signals^[13-17]. In contrast, natural antagonists of PSC activation have not been systematically studied so far. Their elucidation, however, may be helpful for the development of antifibrotic therapies.

Interferons (IFNs) are multifunctional cytokines that block viral infection, modulate immune as well as inflammatory responses, and inhibit cell proliferation^[18]. IFN- α is an effective drug for the treatment of patients with chronic hepatitis B or C associated with liver fibrosis^[19,20]. Recent studies suggest that IFNs not only block virus replication but also directly affect key functions

of activated HSCs. Thus, IFN- γ inhibits HSC survival, cell proliferation and collagen synthesis^[21-23]. The effects of IFNs on PSCs are largely unknown.

IFNs exert their biological effects on target cells by binding to cell surface receptors with specificity for either type I interferons (IFN- α , - β , and - ω) or type II interferon IFN- γ ^[18,24]. In the transduction of signals from activated IFN-receptors to the nuclei, tyrosine kinases of the Janus family (JAKs) and signal transducer and activator of transcription (STAT) transcription factors play a key role^[18,25]. IFN- α activates the JAK family proteins (JAK1 and TYK2) as well as the ISGF3 complex (consisting of STAT1, 2 and the IFN regulatory family protein p48)^[26]. Although tremendous progress has been made, the molecular mechanisms underlying the growth-inhibitory action of these cytokines are only partially characterized so far.

This study was to analyze the biological effects of IFN- α , - β and - γ on PSCs and to study the molecular determinants of IFN efficiency.

MATERIALS AND METHODS

Materials

The enhanced chemiluminescence (ECL) plus kit, horseradish-peroxidase labeled antibodies and [³H]-proline were purchased from Amersham Biosciences (Freiburg, Germany), the phospho-STAT1 and STAT3 antibodies from New England Biolabs (Frankfurt, Germany), and anti-STAT1 and STAT3 protein antibodies from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Hank's buffered salt solution (HBSS), Iscove's modified Dulbecco's medium (IMDM) and all supplements for cell culture were obtained from Biochrom (Berlin, Germany), Nycodenz from Nycomed (Oslo, Norway), simvastatin from Merck Biosciences (Schwalbach, Germany) and the recombinant rat interferons from R&D Systems (Minneapolis, MN, USA). Ascorbat, β -aminopropionitrile and the α -SMA antibody as well as standard laboratory chemicals were from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

PSCs were isolated from the pancreas of male LEW.1W inbred rats by collagenase digestion of the organ and Nycodenz (120 g/L) density gradient centrifugation as previously described^[13]. PSCs collected from the top of the gradient were washed and resuspended in IMDM supplemented with 170 g/L fetal calf serum (FCS), 10 mL/L non-essential amino acids (dilution of a 100 \times stock solution), 10⁵ U/L penicillin and 100 mg/L streptomycin. The cells were cultured at 37°C in a 50 mL/L CO₂ humidified atmosphere. All experiments were performed with cells growing in primary culture, or depending on the experimental settings, with cells of the first passage. If replating of the cells was required, PSCs were harvested by trypsinization on d 7 after isolation and recultured at equal seeding densities.

Quantification of DNA synthesis

To quantitate cell proliferation, incorporation of

5-bromo-2'-deoxyuridine (BrdU) into newly synthesized DNA was measured using the BrdU labeling and enzyme-linked immunosorbent assay kit (Roche Diagnostics). Therefore, cells were plated in 96-well plates in complete culture medium supplemented with IFN as indicated. After 48 h, BrdU labeling was initiated by adding labeling solution at a final concentration of 20 mL/L. After another 16 h, labeling was stopped, and BrdU uptake was measured according to the manufacturer's instructions.

Analysis of cellular DNA content by flow cytometry (sub-G₁-peak)

Cells were grown to about 80% confluency and then incubated for 48 h in complete culture medium with or without IFN as indicated. Then the cells were harvested by trypsinization, washed twice with PBS and resuspended in 2 mL ice-cold 70% ethanol for at least 12 h at -20°C, washed again twice with PBS and incubated for 1 h in 400 μ L PBS containing 1 mL/L Tween 20 and 1 g/L RNase at room temperature. After the addition of 50 μ g propidium iodide/10⁶ cells, samples were subjected to cytofluorometric analysis using an FACScan (Becton Dickinson) and the CellQuest program. Ten thousand events were measured for each sample and the data were stored in list mode for further analysis.

Quantification of collagen synthesis

Collagen synthesis was assessed through the quantification of [³H]-proline incorporation into acetic acid-soluble proteins. Therefore, cells were plated in 12-well plates and grown to subconfluency. Afterwards, they were cultured for 48 h in complete culture medium supplemented with 2.5 mCi/L [³H]-proline (48 Ci/mmol), 50 mg/L ascorbate, 50 mg/L β -aminopropionitrile and IFN as indicated. All further steps were performed essentially as previously described^[27]. Raw data of [³H]-proline incorporation were normalized on the basis of absolute cell counts determined by trypan blue staining of PSCs cultured in parallel under identical conditions, except that no [³H]-proline was added.

Immunoblotting

Protein extracts of PSCs (pretreated as indicated) were prepared, adjusted to identical protein concentrations and subjected to immunoblot analysis as previously described^[13]. Briefly, proteins (15 μ g/sample) were separated by SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose membrane. Next, the filters were blocked with 1% bovine serum albumin (Sigma-Aldrich) and incubated with the indicated antibodies (diluted according to the manufacturer's instructions) overnight at 4°C. After a final incubation with a horseradish-peroxidase labeled anti-rabbit or anti-mouse Ig antibody for 2 h at room temperature, blots were developed using ECL. For reprobing with additional antibodies, membranes were stripped by incubation in stripping buffer (62.5 mmol/L Tris-HCl, pH 6.7, 20 g/L SDS, 100 mmol/L 2-mercaptoethanol) at 50°C for 30 min.

Inhibition of STAT1 expression using siRNA

STAT1 siRNA was purchased from Qiagen (Hilden,

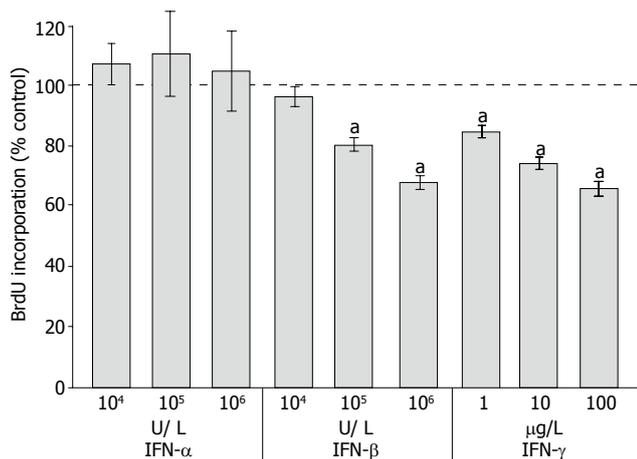


Figure 1 Effects of interferons on PSC proliferation. PSCs growing in primary culture were harvested, replated at equal seeding densities in 96-well plates and treated with IFN- α , IFN- β and IFN- γ at the indicated concentrations for 48 h, the time when the fastest-growing cultures were almost confluent. Cell proliferation was assessed with the BrdU DNA-incorporation assay. One hundred percent cell proliferation corresponds to untreated PSCs. Data are presented as mean \pm SE ($n=6$ separate cell preparations from one rat). ^a $P<0.05$ vs control cultures. The results shown are representative of three experiments using cells from different rats.

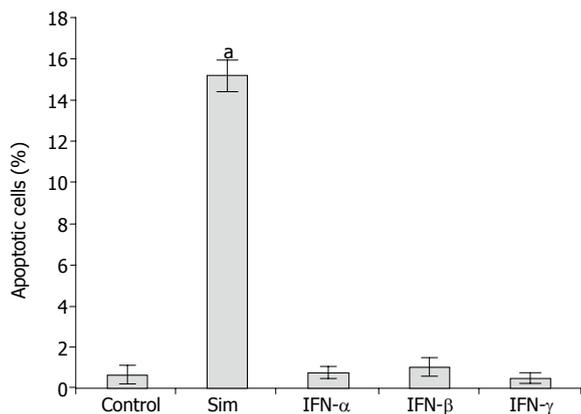


Figure 2 Effects of IFN and simvastatin on PSC survival. PSCs growing in 12-well plates were cultured with IFN- α (10⁵ U/L), IFN- β (10⁶ U/L), IFN- γ (100 μg/L) and simvastatin (Sim; 5 μmol/L) as indicated. To determine the percentage of cell death by flow cytometry analysis, cells with DNA content lower than the G₁ peak (sub-G₁) were considered apoptotic. Data are indicated as mean \pm SE ($n=6$ separate cell preparations from one rat). ^a $P<0.05$ vs control cultures. Three experiments using cells from different rats yielded qualitatively identical results.

Germany) and applied according to the manufacturer's protocol for the transfection of adherent cells, using a 1:6 ratio of siRNA to RNAsFect Reagent. Efficiency of siRNA treatment was monitored by analyzing the STAT1 protein level at different times after siRNA application. To study the effects of STAT1 siRNA on PSC growth, cells growing in 96-well plates were pretreated with siRNA at 100 nmol/L for 24 h before IFN- γ was added as indicated. After 48 h of incubation, DNA synthesis was quantitated by the determination of BrdU incorporation into DNA as described above. In all the experiments, a non-silencing siRNA oligonucleotide was included as a negative control.

Statistical analysis

Results were expressed as mean \pm SE for the indicated

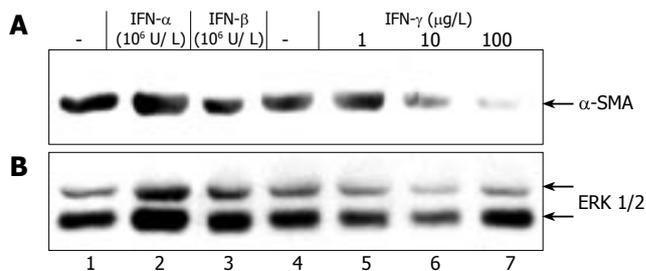


Figure 3 IFN- γ inhibits expression of α -SMA in PSCs. Freshly isolated PSCs growing in 6-well plates were treated with IFN for 9 d as indicated. Cell lysates were normalized for protein concentration and resolved by SDS-PAGE. **A:** Expression of α SMA assayed by immunoblotting; **B:** Blot stripped and reprobed with an anti-ERK 1/2 protein-specific antibody to control loading. Results are representative of three independent experiments.

number of separate cultures per experimental protocol. Statistical significance was checked using Wilcoxon's rank sum test. $P<0.05$ was considered statistically significant.

RESULTS

Effects of IFNs on PSC functions

To analyze the effects of IFN- α , IFN- β and IFN- γ on PSC proliferation, DNA synthesis after IFN-pretreatment for 48 h was measured (Figure 1). Both IFN- β and IFN- γ inhibited DNA synthesis significantly and in a dose-dependent manner, causing more than 30% reduction at the highest concentration tested. In contrast, IFN- α even at the concentration of 10⁶ U/L had no effect on PSC growth. To study if the inhibition of DNA synthesis was in fact due to the induction of apoptosis, an FACS analysis was performed (Figure 2). Under the experimental conditions tested, none of IFN- α , IFN- β or IFN- γ induced a significant increase of the sub-G₁ fraction (representing apoptotic cells). In contrast, PSCs treated with simvastatin significantly accumulated in the sub-G₁ fraction, confirming our previous results of pro-apoptotic effects of hydroxymethylglutaryl coenzyme A reductase inhibitors^[28].

Next, we asked how IFNs affect the myofibroblastic phenotype of activated PSCs. IFN- γ incubation for 9 d (starting immediately after isolation) was associated with a dose-dependent reduction of α -SMA protein expression (Figure 3), while the effect of IFN- β (at 10⁶ U/L) remained questionable and IFN- α did not display any inhibitory effect at all. Further studies revealed that IFN- γ (at ≥ 1 μg/L) and IFN- β (at $\geq 10^4$ U/L), but not IFN- α (at 10⁶ U/L) significantly diminished collagen synthesis by PSCs (Figure 4). A further increase of IFN- γ and IFN- β concentrations had no additional effect (data not shown).

To correlate the biological effects of the three IFNs with their molecular action, we analyzed STAT protein activation in IFN-stimulated PSCs (Figure 5). IFN- β and IFN- γ induced a rapid and strong increase of STAT1 and STAT3 tyrosine phosphorylation, while IFN- α was much less effective.

In subsequent experiments, we further studied the role of STAT1 in the mediation of IFN- γ effects on

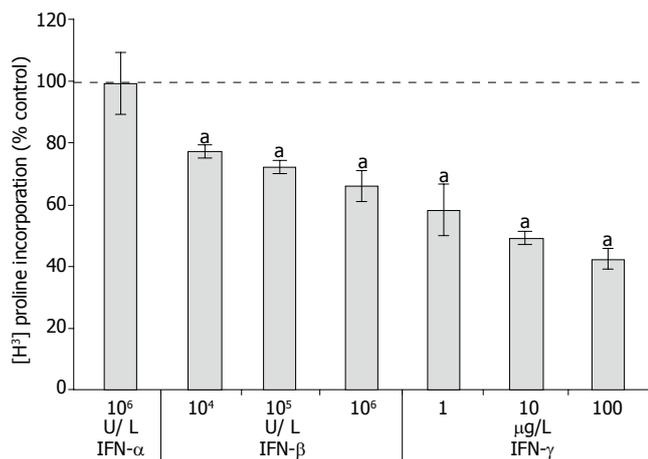


Figure 4 Effects of IFNs on [³H]-proline incorporation in PSCs. PSCs growing in 12-well plates (one passage) were exposed for 48 h to IFNs as indicated. Collagen synthesis was analyzed through the quantification of [³H]-proline incorporation into acetic acid-soluble proteins, and raw data were normalized for differences in cell growth rates as described under "Materials and methods". One hundred percent [³H]-proline incorporation corresponds to untreated PSCs. Data are presented as mean ± SE (n=6 separate cell preparations from one rat). *P<0.05 vs control cultures. The results shown are representative of three experiments using cells from different rats.

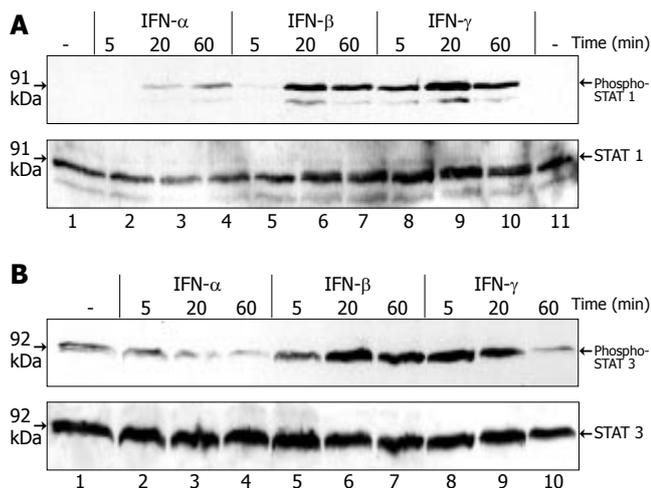


Figure 5 Tyrosine phosphorylation of STAT1 and STAT3 in PSCs induced by IFN. PSCs growing in 6-well plates (one passage) were treated with IFN-α (10⁶ U/L), IFN-β (10⁶ U/L) and IFN-γ (100 μg/L) as indicated. Cell lysates were normalized for protein concentration and resolved by SDS-PAGE. **A:** Tyrosine phosphorylation of STAT1 assayed by immunoblotting using an antibody specific for the tyrosine-phosphorylated protein (upper panel). To control loading, the blot was stripped and reprobed with an anti-STAT1 protein-specific antibody (lower panel); **B:** Immunoblot analysis performed using an antibody specific for tyrosine-phosphorylated STAT3 (upper panel) and the blot stripped and reprobed with an anti-STAT3 protein-specific antibody (lower panel). Results are representative of three independent experiments.

PSC growth. As shown in Figure 6A (upper panel), STAT1 siRNA treatment caused a marked decrease of the STAT1 protein level. Expression of ERK 1 and 2 remained unchanged (lower panel), suggesting that the siRNA inhibited STAT1 expression in a specific manner. Downregulation of the STAT1 protein level was associated with a significant attenuation of the growth-inhibitory effect of IFN-γ (Figure 6B).

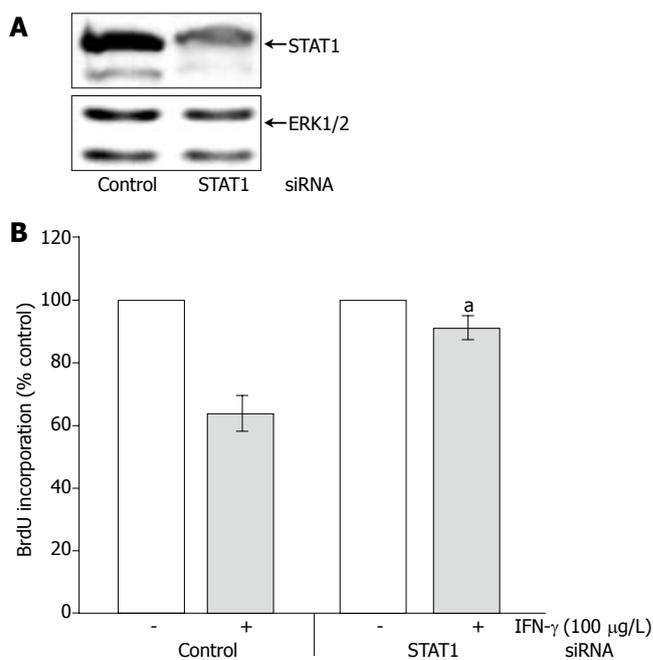


Figure 6 Effects of STAT1 siRNA on PSC growth. **A:** Expression of STAT1 in PSCs 5 d after STAT1 siRNA application determined by immunoblot analysis as well as ERK 1 and 2 expression analyzed using an anti-ERK 1/2 protein-specific antibody; **B:** PSCs treated with STAT1 siRNA and IFN-γ as indicated and control cultures were exposed to a non-silencing control oligonucleotide at the same concentration. PSC proliferation was analyzed through the determination of BrdU incorporation into newly synthesized DNA as described in the section "Materials and methods". One hundred percent cell proliferation corresponds to PSCs treated with control oligonucleotide (left two columns) or STAT 1 siRNA (right two columns) in the absence of IFN-γ. Data are presented as mean ± SE (n=7 separate cell preparations from one rat). *P<0.05 vs IFN-γ-treated control cultures. The results shown are representative of three experiments using cells from different rats.

DISCUSSION

Chronic pancreatitis is associated with an extended fibrosis that contributes to the development of an exocrine and endocrine insufficiency of the gland. Excessive deposition of connective tissue is also typical of pancreatic cancer and has been implicated in the acceleration of tumor progression^[29]. In both cases, activated PSCs have been identified as the principle ECM-producing cell type^[3,7,30]. PSCs are therefore considered as a promising target for the development of adjuvant therapies aimed at inhibiting pancreatic fibrogenesis.

The results of this study showed that IFNs displayed direct inhibitory effects on key effector functions of activated PSCs. While both IFN-γ and IFN-β diminished PSC proliferation and collagen synthesis, only IFN-γ efficiently reduced the expression of α-SMA, an indicator of myofibroblastic transdifferentiation. The effects of IFN-α remained insignificant. Whether the latter observation could reflect a peculiarity of the *in vitro* model used in this study, or a general phenomenon of PSC biology, is currently unclear.

The findings described here are largely in agreement with the results of studies on hepatic stellate cells which also showed direct, but distinct inhibitory effects of different IFNs on stellate cell activation. Shen *et al.*^[21] observed that HSC proliferation is attenuated by IFN-β

and IFN- γ , but not by IFN- α . On the other hand, under the experimental conditions used in this study no significant effects of IFNs on cell survival were detected, while in HSCs pro-apoptotic effects of IFN- γ and an anti-apoptotic action of IFN- α have been reported^[23].

Targeted gene disruption studies have shown that the transcription factor STAT1 plays an essential, non-redundant role in IFN-mediated innate immunity to viral diseases^[31,32]. Analyzing possible functions of STAT proteins in PSCs, we found a correlation between the efficiency of the different IFNs to inhibit stellate cell functions and their potential to activate STAT1 (as well as STAT3). Further experiments using an siRNA approach to block STAT1 expression revealed a direct involvement of the transcription factor in PSC growth inhibition by IFN- γ . The precise functions of STAT3 in PSCs remain to be characterized.

Previous studies in our laboratory have shown that IFN- γ in PSCs stimulates the expression of interleukin (IL)-15. In a co-culture model of rat PSCs and lymphocytes, a suppression of spontaneous lymphocyte apoptosis has been observed, which is at least in part mediated by IL-15^[33]. Together, these data and the results of this study suggest a complex role of IFN- γ in the modulation of pancreatic inflammation and fibrosis. It is conceivable that IFN- γ displays distinct net effects on the progression of chronic pancreatitis, depending on the activation of auto-reactive lymphocytes and possibly the stage of the disease. To further elucidate the effects of IFN- γ on pancreatic fibrogenesis, *in vivo* studies using animal models of pancreatic fibrosis are required.

In summary, the discovery of direct inhibitory IFN-effects on PSC activation encourages further studies with respect to IFN efficiency in chronic pancreatitis. Currently, we are also focusing on a systematic evaluation of IFN target genes in stellate cells.

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BASIC RESEARCH

Characterization of human gene encoding SLA/LP autoantigen and its conserved homologs in mouse, fish, fly, and worm

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Abstract

AIM: To approach the elusive function of the SLA/LP molecule, we have characterized genomic organization and conservation of the major antigenic and functional properties of the SLA/LP molecule in various species.

METHODS: By means of computational biology, we have characterized the complete SLA/LP gene, mRNA and deduced protein sequences in man, mouse, zebrafish, fly, and worm.

RESULTS: The human SLA/LP gene sequence of approximately 39 kb, which maps to chromosome 4p15.2, is organized in 11 exons, of which 10 or 11 are translated, depending on the splice variant. Homologous molecules were identified in several biological model organisms. The various homologous protein sequences showed a high degree of similarity or homology, notably at those residues that are of functional importance. The only domain of the human protein sequence that lacks significant homology with homologous sequences is the major antigenic epitope recognized by autoantibodies from autoimmune hepatitis (AIH) patients.

CONCLUSION: The SLA/LP molecule and its functionally relevant residues have been highly conserved throughout the evolution, suggesting an indispensable function of the molecule. The finding that the only non-conserved domain is the dominant antigenic epitope of the human SLA/LP sequence, suggests that SLA/LP autoimmunity is autoantigen-driven rather than being driven by molecular

INTRODUCTION

Autoantibodies to soluble liver antigen (SLA)^[1] and to liver/pancreas (LP)^[2] have been described as specific markers for autoimmune hepatitis^[3]. Recently, identity of the SLA and LP antigens has been demonstrated, and the target antigen of SLA/LP autoantibodies has been cloned^[4]. Using the recombinant SLA/LP molecule, the strict specificity of SLA/LP autoantibodies as markers for AIH has been confirmed^[5].

The primary biological function of SLA/LP remains unclear. Because the SLA/LP molecule was found to be associated with the UGA tRNP(Ser)Sec complex^[6,7], which facilitates the co-translational incorporation of selenocysteine into proteins, it has been speculated that the SLA/LP molecule may have a role in selenoprotein metabolism; the specialized UGA tRNA is initially charged with serine to form seryl-tRNA, which then is enzymatically converted to selenocysteine-tRNA^{Sec}. However, there is no direct experimental evidence for such a role of the SLA/LP molecule so far. Nevertheless, a fold recognition study predicted the SLA/LP tertiary structure by comparison to known protein structures to be that of a pyridoxal phosphate (PLP)-dependent transferase^[8], which is compatible with a role in selenoprotein metabolism. The active site was proposed to be a cavity with a channel, formed by dimerization of two SLA/LP molecules^[8,9]. In the three-dimensional model, five amino acids of monomer A (L88, T89, F92, T94, and S118) as well as two amino acids of monomer B (P251 and G252) were involved in dimerization and the amino acids critical for binding and orientation of the co-enzyme PLP were

identified to be G50, S51, S165, D186, H189, K224^[8] (residue numbering according to GenBank accession number NP_722547).

The immune mechanisms that drive SLA/LP autoimmunity and the pathogenetic relevance of autoimmunity to SLA/LP are currently not clear. The main antigenic region crucial for the recognition of SLA/LP autoantibodies was identified to locate between amino acids 390 and 428^[4,10], thus being easily accessible by antibodies^[8]. This region contains sequence homologies with various microbial antigens, including proteins of Rickettsia species, human herpesvirus 6, and cytomegalovirus^[4]; however, the homologous microbial sequences are only poorly recognized by SLA/LP autoantibodies^[10]. Nevertheless, molecular mimicry of homologous proteins from other species, such as parasites, may be a trigger of SLA/LP autoimmunity.

Due to the human genome project and other genomic sequencing groups, sequences of many different species are now publicly available. While some chromosomes are completely assembled, most genomic fragments are currently still preliminary and await characterization. To approach the elusive biological function of the SLA/LP molecule, we have characterized here the human SLA/LP gene and homologous sequences of other species by means of computational biology. The obtained results support the hypothesis that SLA/LP autoimmunity is autoantigen-driven rather than being driven by molecular mimicry^[10].

MATERIALS AND METHODS

Identification of the human SLA/LP gene

We have previously identified the human SLA/LP protein and deposited the corresponding mRNA sequence into the GenBank^[11] with the accession numbers NM_016955 and NM_153825. BLASTing the human mRNA sequence against the human genome draft sequence revealed the genomic localization^[12,13].

Identification of SLA/LP homologs in other species

Homologs corresponding to the human SLA/LP were identified by BLAST query with the human SLA/LP protein sequence (NP_722547) against the non-redundant databases of mouse (*Mus musculus*), zebrafish (*Danio rerio*), fruit fly (*Drosophila melanogaster*) and the nematode worm (*Caenorhabditis elegans*).

Exon/intron structure and chromosomal localization

Exon/intron structure and chromosomal localization were identified by BLASTing the SLA/LP mRNA sequences of each homolog against the corresponding genome draft sequences of the particular species.

Locus analysis

Data regarding the genomic loci analysis were obtained from the GenBank^[11].

Analysis of protein structure and conservation

The amino acid sequence was deduced from the

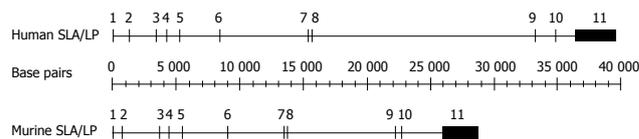


Figure 1 Organization of SLA/LP genes in man and mouse. The exon/intron structure of the human and mouse SLA/LP gene is represented schematically. Exon sequences (numbered boxes) are highly conserved and of similar size and number in man and mouse. Intron sequences, marked as lines, differ in both species.

obtained mRNA sequences. The alignment of SLA/LP homologous sequences was initially performed using the ClustalW algorithm^[14]. The sequence alignments in Figure 3 were performed using the longer human SLA/LP variant (CAB89517). The theoretical isoelectric point and molecular weight was calculated with the pI/Mw tool on the Expasy server^[15,16]. Protein structures and domains were analyzed using two independent algorithms, Pfam^[17] and NCBI Conserved Domain Search^[18].

RESULTS

Human SLA gene and corresponding mRNA and protein sequences

We have previously submitted the human mRNA sequences of two alternative splice variants of SLA/LP to GenBank with the accession numbers NM_016955 and NM_153825. Using the human SLA/LP protein sequence as a query for BLAST searches against the human mRNA database, additional matching mRNA sequences (splice variants) were not identified. Matching the human mRNA sequence to the available human genomic draft sequence (htgs), the corresponding gene was mapped to chromosome 4p15.2. The gene was found to span a genomic region of approximately 39 kb and to be organized in 11 exons (Figure 1). Most exon sizes were rather small, ranging between 91 and 187 bp, with the exception of exon 11 at the 3' end, which covered 3 224 bp. The two splice variants were fully identical, except for the absence of exon 2 (149 bp) in the shorter variant. Both variants have a large 2 930 bp 3' untranslated region; the 5' untranslated region of the two variants is relatively short with 155 and 174 bp, respectively. The amino acid sequences of the two splice variants, as deduced from their mRNA sequence, differ only in their amino-terminal residues (Figure 2). The theoretical molecular weight and isoelectric point of the longer splice variants is 48.84 kDa and 8.64, respectively; that of the shorter variant is 46.85 kDa and 8.52, respectively.

Mouse SLA gene and corresponding mRNA and protein sequences

The SLA/LP gene and genomic organization was highly conserved in mouse. BLASTing the human SLA/LP protein sequence against the mouse protein database, revealed a mouse homolog with the accession number NP_766078. The human and mouse SLA/LP protein sequences are identical at 85% of the residues (Figure 2). Both, the NCBI and Pfam conserved protein domain

| | |
|-------------|----------------------------------|
| Human short | MS - TSYGCFWRR 11 |
| Mouse short | MNPESF AAGE RR 12 |
| | * . * . . * |
| Human long | MDSNNFLGNCVGEREGRVASALVARRHYR 30 |
| Mouse long | MDSNNFLGNCVGEREGRVASALVARRHYR 30 |
| | ***** |

| | | |
|--------------|--|---------|
| Human SLA/LP | FIHGIGRSGDISAVQPKAAGSSLLNKITNSLVLD I I K L A G V H T V A N C F V V P M A T | 65/84 |
| Mouse SLA/LP | FIHGIGRSGDISAVQPKAAGSSLLNKITNSLVLD I I K L A G V H T V A N C F V V P M A T | 66/84 |
| | ***** | |
| Human SLA/LP | GMSLTLCLFLTLRHKRPKAKYIHWPRIDQKSCFKSMI TAGFEPVVIENVLEGDEL | 119/138 |
| Mouse SLA/LP | GMSLTLCLFLTLRHKRPKAKYIHWPRIDQKSCFKSMVTAGFEPVVIENVLEGDEL | 120/138 |
| | ***** | |
| Human SLA/LP | RIDLKAVEAKVQELGPDCLIC I H S T T S C F A P R V P D R L E E L A V I C A N Y D I P H I V N | 173/192 |
| Mouse SLA/LP | RIDLKAVEAK I Q E L G P E H I L C L H S T T A C F A P R V P D R L E E L A V I C A N Y D I P H V V N | 174/192 |
| | ***** | |
| Human SLA/LP | NAYGVQSSKCMHLIQQGARVGRIDAFVQSLDKNFMPVPGGAIAGFNDSFIQEI | 227/246 |
| Mouse SLA/LP | NAYGLQSSKCMHLIQQGARVGRIDAFVQSLDKNFMPVPGGAIAGFNDFIQDI | 228/246 |
| | ***** | |
| Human SLA/LP | SKMYPGRASASPSLDVLTLLSLGCSGYRLLKERKEMFVYLSYLSNQI KKLSEA Y N | 281/300 |
| Mouse SLA/LP | SKMYPGRASASPSLDVLTLLSLGCSGYRLLKERKEMFVYLSYLSNQI KKLSEA Y N | 282/300 |
| | ***** | |
| Human SLA/LP | ERLLHTPHNPISLAMTLKTLDEHRDKAVTQLGSMFLTRQVSGARVPLGSMQTV | 335/354 |
| Mouse SLA/LP | ERLLQTPHNPISLAMTLKTLDEHRDKAVTQLGSMFLTRQVSGARVPLGNVQTV | 336/354 |
| | ***** | |
| Human SLA/LP | SGYTFRGFMSHTNNYP CAYLNA A S A I G M K M Q D V D L F I N R D R C L K A V R K E R S K E | 389/408 |
| Mouse SLA/LP | SGHTFRGFMSHADNYP CAYLNA A A I G M K M Q D V D L F I K R L D K C L N I V R K E Q T R A | 390/408 |
| | ***** | |
| Human SLA/LP | ---SDDNYDK T E D V D I E E M A L K L D N V L L D T Y Q D A S S | 422/441 |
| Mouse SLA/LP | SVVSGADRNKAEADIEEMALKLDDVLDVGVQGPAL | 426/444 |
| | ***** | |

Figure 2 SLA/LP splice variants in man and mouse. The amino acid sequences of the long and short SLA/LP splice variants in humans and their putative homologs in mouse are aligned. The protein variants differ only in their amino-terminal sequence, 11 or 30 residues in the human protein and 12 or 30 residues in the murine protein, as depicted in the upper panel. The other residues (lower panel) are identical in the respective splice variants within each species.

| | | |
|---------------|---|-----|
| Homo sapiens | ---MDSNNFLGNCVGEREGRVASALVARRHYRF I HGIGRSGD IS AVQPKAAGSSLLNK | 56 |
| Mus musculus | ---MDSNNFLGNCVGEREGRVASALVARRHYRF I HGIGRSGD IS AVQPKAAGSSLLNK | 56 |
| Danio rerio | ---MDSNNFLGNCVGEREGRVASALVARRHYRL I HGIGRSGD IAAVQPKAAGSSLLNK | 56 |
| Drosophila m. | MLASLDSNNYP HKVGLGEREAR I ACKLVARRHYNFGHGIGRSGDLL EAQPKAAGSTLLAR | 60 |
| C. elegans | -----MIPV G A G E R E G R V L T P L V Q R L H S N L T H G I G R S G N L L E I Q P K A L G S S M L A C | 50 |
| | * * * * * * * * * * * * * * * * * * * * | |
| Homo sapiens | I T N S L V L D I I K L A G V H T V A N C F V V P M A T G M S L T L C F L T L R H K R P K A K Y I I W P R I D Q K S C F | 116 |
| Mus musculus | I T N S L V L N V I K L A G V H S V A S C F V V P M A T G M S L T L C F L T L R H K R P K A K Y I I W P R I D Q K S C F | 116 |
| Danio rerio | I T N S V L V D V L K L T G V R S V S C F V V P M A T G M S L T L C F L T L R H R R P K A R Y I L W P R I D Q K S C F | 116 |
| Drosophila m. | L T N A L I L D L I R G I G L P S C A G C F L V P M C T G M T L T L C Q S L R K R R P G A R Y V L W S R I D Q K S C F | 120 |
| C. elegans | L S N E F A K H A L H L L G L H A V K S C I V V P L C T G M S L S L C M T S W R R R R P K A K Y V V W L R I D Q K S S L | 110 |
| | ::* * * * * * * * * | |
| Homo sapiens | K S M I T A G F E P V V I E N V L E G D E L R T D L K A V E A K V Q E L G P D C I L C I H S T T S C F A P R V P D R L E | 176 |
| Mus musculus | K S M V T A G F E P V V I E N V L E G D E L R T D L K A V E A K I Q E L G P E H I L C L H S T T A C F A P R V P D R L E | 176 |
| Danio rerio | K S M V T A G F E P V V I E N V L E G D E L R T N L E E V E R K I E E F G A E N T L C V H S T T S C F A P R V P D R L E | 176 |
| Drosophila m. | K A I T A T G L V P V I P C L I K G E S L N T N V D L F R E K I K S L G V D S I L C L Y T T S C F A P R N S D D I A | 180 |
| C. elegans | K S I Y H A G F E P I I V E P I R D R D S L I T D V E T V N R I I E Q R G E E - I L C V M I T T S C F A P R S P D N V E | 169 |
| | * * * * * * * * * | |
| Homo sapiens | E L A V I C A N Y D I P H I V N N A Y G V Q S S K C M H L I Q Q G A R V G R I D A F V Q S L D K N F M P V P G G A I I A | 236 |
| Mus musculus | E L A V I C A N Y D I P H V N N A Y G L Q S S K C M H L I Q Q G A R V G R I D A F V Q S L D K N F M P V P G G A I I A | 236 |
| Danio rerio | E L S V L C A K H D I P H I V N N A Y G V Q P S K C M H L I Q Q G A R V G R I D A F V Q S L D K N F V P V P G G A I I A | 236 |
| Drosophila m. | E V S K L S K W Q I P H L V N N A Y G L Q A K E I V N Q L E C A N R V G R I D Y F V Q S S D K N L L V P V G S A I V A | 240 |
| C. elegans | A I S A I C A A H D V P H L V N N A Y G L Q S E E T I R K I A A A H E C G R V D A V V Q S L D K N F Q V P V G G A I A | 229 |
| | ::* * * * * * * * * | |
| Homo sapiens | G F N D S F I Q E I S K M Y P G R A S A S P S L D V L I T L L S L G S N G Y K L L K E R K E M F S Y L S N Q I K K L S | 296 |
| Mus musculus | G F N E P F I Q D I S K M Y P G R A S A S P S L D V L I T L L S L G S G Y R K L L K E R K E M F V Y L S T Q L K K L A | 296 |
| Danio rerio | G F D E N F I Q E I S K I Y P G R A S A S P S L D V L I T L L T L G A N G Y K L L A D R K E L Y G H L A Q E L S A L A | 296 |
| Drosophila m. | S F N E S V L H D V A S T Y A G R A S G S Q S L D V L M T L L S L G R N G F R L L F D Q R G E N F N Y L R E N L R K F A | 300 |
| C. elegans | A F K Q N H I Q S I A Q S Y P G R A S V P S R D L V L T L L Y Q G Q S A F L E P F G K Q K Q M F L K M R R K I S F A | 289 |
| | . * * * * * * * * * | |
| Homo sapiens | E A Y N E R L L H T P H N P I S L A M T L K T L D E H R D K A V T Q L G S M L F T R Q V S G A R V V P L G S M Q - T V S | 355 |
| Mus musculus | E A H N E R L L Q T P H N P I S L A M T L K T I D G H H D K A V T Q L G S M L F T R Q V S G A R A V P L G N V Q - T V S | 355 |
| Danio rerio | A R H G E R L L K T P H N P I S L A M S L N H L E A H S S A V T Q L G S M L F T R Q V S G A R V V P L G V Q Q - T V S | 355 |
| Drosophila m. | E P R G E I V I D S R F N S I S L A I T L A T L A G D Q M K S I T K L G S M L H M R G V S G A R V I V P G Q N K - T I D | 359 |
| C. elegans | E N I G E C V Y E P E N E I S S A M T L S T I P P A K Q - - - T L F G S I L F A K G I T G A R V V T S S Q S K T T I E | 346 |
| | * * * * . . . * * * * * * * | |
| Homo sapiens | G Y T F R G F M S H T N N Y P C A Y L N A A S A I G M K M Q D V D L F I K R L D R C L K A V R K E R S K E - - - S D D N | 412 |
| Mus musculus | G H T F R G F M S H A D N Y P C A Y L N A A A I G M K M Q D V D L F I K R L D K C L N I V R K E Q T R A S V S G A D | 415 |
| Danio rerio | G H T F S G F M S H S E A Y P C P Y L N A A S A I G I T K G D V I V C M K R L G K C L K I L K E K S D P - - - - A | 409 |
| Drosophila m. | G H E F L G K - - - - - | 366 |
| C. elegans | G C E F I N F G S H T T E Q H G G Y L N I A C S V G M T D H E L E E L F T R L T S S Y A K F V R E L A K E - - - D E R I | 403 |
| | * * * * * * * * * | |
| Homo sapiens | Y D K T E D V D I E E M A L K L D N V L L D T Y Q D A S S | 441 |
| Mus musculus | R N K A E D A D I E E M A L K L D D V L D V G V Q G P A L | 444 |
| Danio rerio | D L E A E D G E L E S P Q R S T E L R V - - - - - | 430 |
| Drosophila m. | - - - - - | |
| C. elegans | N S S G R R I P I N E S F D M E N D - - - - - | 421 |

Figure 3 Alignment of SLA/LP protein sequences. The SLA/LP amino acid sequences of various eukaryotic species were aligned by ClustalW. The presumed residues that mediate binding to the cofactor pyridoxal phosphate are highlighted in bold and underlined; residues that function in dimerization are highlighted in bold and italic. The immunodominant epitope sequence (390-428) of the human SLA/LP protein is marked in bold.

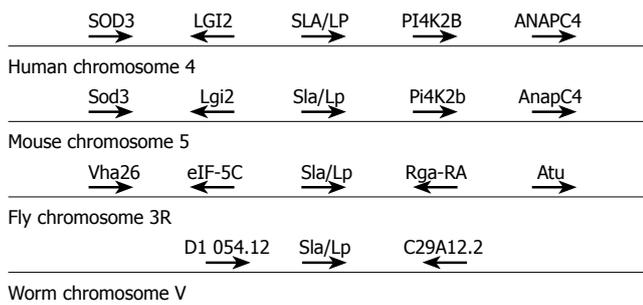


Figure 4 Chromosomal localization of SLA/LP genes. The genes adjacent to the SLA/LP loci on human chromosome 4, mouse chromosome 5, fly chromosome 3R, and worm chromosome V are represented schematically.

algorithms, predicted the existence of SLA/LP structure between amino acids 61 and 458 and amino acids 61 and 459, respectively. BLASTing the protein sequence against the translated mouse genome database revealed the corresponding mRNA NM_172490.1. BLASTing the mRNA sequence against the draft sequence of the mouse genome, the corresponding SLA/LP gene was mapped to mouse chromosome 5qC1. The genomic region spanned 28.5 kb and the mouse SLA/LP gene was organized into 11 exons like the human homolog (Figure 1). Exon sizes ranged between 91 and 2 927 bp with exon 11 being by far the largest exon. The 3' untranslated region was 2 629 bp and the 5' untranslated region was 19 bp. Although not filed in the GenBank, two murine splice variants are very likely; the mouse genomic organization and exon sequences are very similar to the human gene and a splicing signature similar to that in human beings is present in the homologous mouse sequence. The presumed splice variants in the mouse are shown in Figure 2, aligned to the corresponding human proteins. The theoretical molecular weight and isoelectric point of the longer splice variants is 48.48 ku and 8.86, respectively; that of the shorter variant is 46.5 ku and 8.71, respectively.

Zebrafish SLA gene sequence and corresponding mRNA

The SLA gene and genomic organization was highly conserved in fish. BLASTing the human SLA/LP protein sequence against the zebrafish protein database, revealed a homolog with the accession number NP_956448. The zebrafish sequence was identical to the human protein sequence in 69% of the residues. Both, the NCBI and Pfam conserved protein domain algorithms, predicted the existence of SLA/LP structure between amino acids 61 and 459. BLASTing the protein sequence against the translated zebrafish genome database revealed the corresponding mRNA NM_200154. Chromosomal mapping was not possible, since the genomic fragment containing the SLA/LP sequence was not yet assembled into the genomic context. The corresponding gene could only be mapped to the fragment scaffold Zv4_NA8411.1. Thus a complete exon/intron structure was not yet available, and potential splice variants could not be identified.

Drosophila SLA gene sequence and corresponding mRNA

The SLA gene and genomic organization were also

conserved in *Drosophila*. BLASTing the human SLA/LP protein sequence against the *Drosophila* protein database revealed a *Drosophila* homolog (two isoforms) with the accession numbers AAF51994 and AAS65099. The *Drosophila* protein sequence has 42% identity compared to the human protein sequence. However, this may be an underestimation, as it remains unclear whether the recently annotated *Drosophila* SLA/LP sequence is complete. Both, the NCBI and Pfam conserved protein domain algorithms predicted the existence of SLA/LP structure between amino acids 61 and 413 and amino acids 61 and 428, respectively. BLASTing the protein sequence against the translated *Drosophila* genome database revealed the corresponding mRNA NM_141299.1. BLASTing the mRNA sequence against the draft sequence of the fruit fly genome, the corresponding SLA/LP gene was mapped to *Drosophila* chromosome 3R. The genomic region of the currently annotated *Drosophila* SLA/LP gene spanning approximately 1.6 kb was found to be organized into (at least) 4 exons. Exon sizes ranged between 114 and 1 021 bp with exon 3 being by far the largest exon. A short 3' UTR was shown to consist of 115 bp. Thus far, a 5' UTR was not identified.

***C. elegans* SLA/LP gene sequence and corresponding mRNA**

The SLA/LP gene and genomic organization were also conserved in *C. elegans*. BLASTing the human SLA/LP protein sequence against the *C. elegans* protein database revealed similarity to *C. elegans* protein D1054.13 with the accession number CAA98446. The *C. elegans* protein sequence had 42% identity compared to the human protein sequence. Again, both the NCBI and Pfam conserved protein domain algorithms predicted the existence of SLA/LP structure between amino acids 53 and 450. BLASTing the protein sequence against the translated *C. elegans* genome database revealed the corresponding mRNA NM_073360. BLASTing the mRNA sequence against the draft sequence of the *C. elegans* genome, the corresponding SLA/LP gene was mapped to *C. elegans* chromosome V. The genomic region spanned 1.8 kb and the *C. elegans* SLA/LP gene was organized in 7 exons. Exon sizes ranged between 67 bp for exon 3 and 636 bp for exon 5. A rather short 5' UTR contained 43 bp, a 3' UTR could not be found in the currently available sequence. Thus, it remains unclear, whether the currently available sequence represents the complete protein sequence of *C. elegans*.

Conservation of SLA/LP homologs

We aligned the protein sequences of the SLA/LP homologs in human, mouse, fish, fly, and worm (Figure 3). The mammalian sequences were the two closest related sequences. The invertebrate sequences of *Drosophila* and *C. elegans* sequence were most closely related to each other; the zebrafish sequence was equally related to the mammalian and invertebrate sequences.

Chromosomal localization of the human, mouse, fly, and worm SLA/LP genes

We then studied the neighboring genes in man, mouse, fly, and worm (Figure 4). Upstream of the human SLA/

LP sequence on human chromosome 4 was the gene for extracellular-superoxide dismutase 3^[19] and the Leucine rich gene, glioma-inactivated 2^[20]. Downstream of the SLA/LP locus was the phosphatidylinositol 4-kinase type-II beta (PI4K2B)^[21] and anaphase promoting complex, subunit 4^[22]. The same structure was found for mouse chromosome 5 that contains the murine SLA/LP locus. The genomic structure of SLA/LP loci in fly and worm did not share these similarities to the mammalian genomes. The fly SLA/LP homolog was flanked by the gene *eIF-5C*, which is involved in long-term memory^[23] and the gene for a 26 ku E-subunit of the vacuolar ATPase^[24], upstream of the SLA/LP locus. Two currently uncharacterized genes, Rga-RA (account number AAF51992) and Atu (account number AAF51991), are located downstream of the fly SLA/LP gene. The worm SLA/LP sequence was flanked by a gene (5L286) encoding protein of unknown function, upstream, and the serpentine receptor class H (srh-184)^[25], downstream of the SLA/LP locus.

Conservation of the main antigenic region and main structural features of folding and dimerization

The dominant autoepitope of SLA/LP autoantibodies had been identified to locate between amino acids 390 and 428 of the longer human SLA/LP protein sequence variant (NP_722547)^[4,10]. After aligning the homologous protein sequences (Figure 3), we studied the degree of sequence conservation in the immunodominant antigenic region. Human and mouse sequences showed 57% identity and 71% similarity. All other sequences did not display a considerable level of similarity compared to the human epitope sequence.

We then studied the conservation of the residues that seem to function in protein folding and dimerization^[8] (Figure 3). The lysine residue at position 224, which is crucial for covalent binding of the coenzyme PLP (pyridoxal-5'-phosphate) was conserved in all homologous sequences investigated. Of the other five residues directly in contact with PLP, G50, S51, and H189 were identical in all the homologs, whereas S165 was replaced by alanine in mouse and D186 was replaced by glutamine in *Drosophila* and aspartic acid in *C. elegans*. Of the residues of monomer A involved in dimerization, only L88 was conserved in all homologous sequences. T89 was conserved in most species, but replaced by serine in *C. elegans*. F92 was replaced by leucine in *Drosophila* and by methionine in *C. elegans*, and otherwise conserved. T94 was conserved in vertebrates, but replaced by serine in non-vertebrates. S118 was replaced by arginine in *Drosophila*. The contact residues of monomer B, P251 and G252, which mediate dimerization, were conserved in all the species.

DISCUSSION

SLA/LP autoantibodies have been demonstrated to be highly specific markers of autoimmune hepatitis^[4,5]. A role of SLA/LP autoimmunity in the pathogenesis of AIH is likely, at least in a subgroup of AIH patients. However, our current understanding of both, immunogenicity and biological function of the SLA/LP molecule is rather limited. To approach the elusive function and

immunogenicity of SLA/LP, we have characterized the genomic organization and sequence conservation of the human SLA/LP molecule and homologous proteins in other eukaryotic species.

For all homologs, mRNA and genomic sequences were available from the GenBank^[12]. However, in contrast to human and mouse protein and mRNA sequences, which seemed to be complete, some genomic sequences have yet to be completely assembled, and thus did not allow for complete and final characterization of the genomic organization. This was especially the case for fish, where no chromosomal assignment could be made. Nevertheless, genomic characterization in man, mouse, fly, and worm was possible.

The two mammalian SLA/LP genes were similar in organization of the exon/intron structure (Figure 1) and genomic localization (Figure 4); exon/intron structure and genomic localization in fish, fly, and worm was clearly different from the mammalian sequences. Nevertheless, all homologs displayed a high degree of similarity and identity in their amino acid sequences (Figure 3). The similarity between the most distant species - man and worm - was as high as 42%. The highest degree of similarity was found between the human and the mouse amino acid sequences (Figure 2); both species also have a highly similar exon/intron structure in the SLA/LP gene (Figure 1). Moreover, both mammalian species seem to generate similar variant proteins (Figure 2) by differential splicing of exon 2. Whether splice variants exist in non-mammalian species is currently not clear. Previously, the residues, which seem to mediate dimerization and binding of PLP, have been identified by molecular modeling^[8]. These residues are highly conserved among the various species studied (Figure 3). Four of six residues that are crucial for PLP-binding are identical in all the species (G50, S51, H189, K224); the other two residues are homologous (S/A165, D/Q185). Two of the seven residues that mediate dimerization were identical in all the studied species (L88, G252), the other residues were equivalent (T/S89, T/S94, S/A118, P/A251), except for F92, which was conserved in man, mouse and fish, but substituted by leucine in fly and methionine in worm.

The query of human protein databases with the human SLA/LP sequence did not reveal any functional homologs to other proteins and the primary biological function of the SLA/LP protein remains unclear. However, the extraordinary degree of conservation, notably of the presumed functional residues, suggests that the SLA/LP protein has an important biological function that relies on PLP binding. The possible role of the smaller splice variant remains obscure. It may be speculated that the smaller protein variant may act as an inhibitor of the elusive function of the larger variant; the molecular signature of the smaller variant is not compatible with a PLP-dependent transferase, as predicted for the larger variant^[8].

The autoimmune response to SLA/LP is not random, but displays a remarkable degree of uniformity: SLA/LP autoantibodies are of a preferred dominant subtype (IgG1) and recognize the same dominant antigenic region, which was mapped to a carboxy-terminal domain between residues 399 and 428 of the longer splice variant (accession

number NP_722547)^[10]. Thus, SLA/LP autoimmunity has features of a highly selected immune response and seems to be driven by the same mechanism in all the patients. Of note, the antigenic region is located within the only domain of the molecule that was not conserved among the various species (Figure 3). Thus, it appears that SLA/LP autoimmunity is specific for the self-antigen and not for homologous sequences from other eukaryotic species. The possibility that SLA/LP autoimmunity might be driven by homologous proteins from parasites is hence quite unlikely. SLA/LP-homologous proteins are only found in eukaryotes and archaeobacteria, but not in eubacteria^[10]. Nevertheless, a few homologous sequences from bacterial or viral proteins with some degree of similarity to the antigenic epitope of the SLA/LP protein do exist; however, these are not recognized by SLA/LP autoantibodies^[10]. Likewise, the corresponding sequence of an archaeobacterial SLA/LP-homolog is also not recognized by SLA/LP autoantibodies^[10]. Therefore, SLA/LP autoimmunity in patients is very likely driven by the self-SLA/LP molecule rather than by a mechanism that involves molecular mimicry.

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BASIC RESEARCH

Trimetazidine: Is it a promising drug for use in steatotic grafts?

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Abstract

AIM: Chronic organ-donor shortage has led to the acceptance of steatotic livers for transplantation, despite the higher risk of graft dysfunction or nonfunction associated with the ischemic preservation period of these organs. The present study evaluates the effects of trimetazidine (TMZ) on an isolated perfused liver model.

METHODS: Steatotic and non-steatotic livers were preserved for 24 h in the University of Wisconsin (UW) solution with or without TMZ. Hepatic injury and function (transaminases, bile production and sulfobromophthalein (BSP) clearance) and factors potentially involved in the susceptibility of steatotic livers to ischemia-reperfusion (I/R) injury, including oxidative stress, mitochondrial damage, microcirculatory diseases, and ATP depletion were evaluated.

RESULTS: Steatotic livers preserved in UW solution showed higher transaminase levels, lower bile production and BSP clearance compared with non-steatotic livers. Alterations in perfusion flow rate and vascular resistance, mitochondrial damage, and reduced ATP content were more evident in steatotic livers. TMZ addition to UW solution reduced hepatic injury and ameliorated hepatic functionality in both types of the liver and protected against the mechanisms potentially responsible for the poor tolerance of steatotic livers to I/R.

CONCLUSION: TMZ may constitute a useful approach in fatty liver surgery, limiting the inherent risk of steatotic liver failure following transplantation.

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Keywords: Steatotic liver, Ischemia-reperfusion, UW preservation solution

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INTRODUCTION

The mounting number of patients awaiting liver transplantation and the limited pool of donor organs have led to the acceptance of marginal livers such as steatotic livers, for transplantation, despite the higher risk of graft dysfunction or nonfunction which is associated with their ischemic preservation^[1-3].

There is evidence indicating that the composition of preservation solutions is critical for the quality of livers kept for prolonged ischemic periods. University of Wisconsin (UW) preservation solution, considered as the gold standard of such solutions, has proved itself effective in preventing liver damage during cold ischemia and has extended storage time limits^[4,5]. However, irreversible injury occurring after prolonged cold periods (between 16 and 24 h) has also been reported. The main aims of organ preservation, therefore, are striving to prolong organ tolerance^[6,7].

Trimetazidine (TMZ), introduced as an anti-ischemic drug into the heart for over 35 years^[8,9], has also been used to protect kidneys exposed to the prolonged cold ischemia (48 h)^[10,11] and is reported to protect liver against the deleterious effects of warm ischemia^[12,13]. In addition, recently, it has been demonstrated that TMZ pre-treatment reduces liver injury and improves liver regeneration and survival rate in an experimental model of partial hepatectomy under hepatic blood inflow occlusion^[14]. Studies examining the underlying protective mechanisms of TMZ suggest that this drug protects mitochondria in cardiomyocytes and isolated perfused heart by releasing the calcium accumulated in the matrix and by restoring mitochondrial membrane impermeability^[8,15]. TMZ improves energy recovery *in vitro* and *ex vivo* in models of myocardial ischemia^[8,15] and reduces oxidative stress

in the liver under warm ischemia^[12]. Additionally, TMZ improves microcirculatory alterations in isolated perfused rat kidneys^[10]. Taken together, these findings suggest that mitochondria, energy metabolism, oxidative stress, and microcirculation might be important targets through which TMZ exerts its cytoprotective effect. Interestingly, severe mitochondrial damage^[16,17], decreased ATP level^[16,18], increased reactive oxygen species (ROS) production^[19,20], and impaired microcirculation^[21,22] have also been proposed as factors that might leave steatotic livers vulnerable to ischemia–reperfusion (I/R) injury. Taking these previous observations into account, here we report the results of a study aimed at investigating whether the addition of TMZ to the standard preservation solution (UW) protects steatotic liver grafts against the deleterious effects of I/R injury.

MATERIALS AND METHODS

Homozygous (obese, Ob) and heterozygous (lean, Ln) Zucker rats (used as reference group), aged 16–18 wk, were purchased from Iffa-Credo (L'Arbresle, France)^[19,20]. In this study we used isolated perfused rat liver. It is a useful experimental system for evaluating hepatic function, isolated from the influence of other organ systems, undefined plasma constituents, and neural-hormonal effects. Hepatic architectures, microcirculation, and bile production were preserved in this experimental model^[23,24]. All procedures were performed under isoflurane inhalation anesthesia. This study respected the European Union regulations (Directive 86/609 EEC) for animal experiments.

Liver procurement and experimental groups

The surgical technique was performed as described elsewhere^[25]. After cannulation of the common bile duct, the portal vein was isolated, and the splenic and gastroduodenal veins were ligated. The steatotic and non-steatotic livers were flushed and preserved in cold UW solution for 24 h with or without the addition of TMZ (10⁻⁶ mol/L). This dose of TMZ has been shown to be beneficial in various experimental models of I/R^[10,26,27]. At higher concentration, TMZ exerted no protective effect^[28]. TMZ was kindly supplied by the Institut de Recherches Internationales Servier (Courbevoie, France).

Protocol 1: Effect of TMZ on steatotic liver injury after cold ischemia

After 24 h of cold storage, livers from 16 Zucker rats (8 Ln and 8 Ob) preserved in UW solution (UW) and livers from 16 Zucker rats (8 Ln and 8 Ob) preserved in UW solution with TMZ (UW+TMZ) were flushed with Ringer's lactate solution. The aliquots of the effluent flush were sampled for the measurements of cumulative ALT and AST after prolonged ischemia.

Protocol 2: Effect of TMZ on steatotic liver injury after normothermic reperfusion

After 24 h of cold preservation, and in order to account for the period of rewarming during surgical implantation *in vivo*^[29,30], livers from 16 Zucker rats (8 Ln and 8 Ob)

preserved in UW solution and livers from 16 Zucker rats (8 Ln and 8 Ob) preserved in UW+TMZ solution were exposed at 22 °C for 30 min prior to reperfusion. Livers were then connected via the portal vein to a recirculating perfusion system for 120 min at 37 °C^[6,25]. Time 0 was the point at which the portal catheter was satisfactorily connected to the circuit. As previously reported^[25,31], during the first 15 min of perfusion (initial equilibration period), the flow was progressively increased in order to stabilize the portal pressure at 12 mmHg (Pressure Monitor BP-1, Instruments, Inc., Sarasota, FL, USA). The flow was controlled using a peristaltic pump (Minipuls 3, Gilson, France)^[6,25]. The reperfusion liquid consisted of a cell culture medium (William's medium E, BioWhittaker, Spain) with a Krebs-Henseleit-like electrolyte composition enriched with 5% albumin, as oncotic supply. The buffer was continuously ventilated with 95% O₂ and 50 mL/L CO₂ gas mixture. The buffer was subsequently passed through a heat-exchanger (37 °C) and a bubble trap prior to entering the liver^[25,30]. During 120 min of normothermic reperfusion, the effluent fluid was collected at 30 min intervals to measure transaminases. After the initial equilibration period of 15 min, flow rate and vascular resistance were assessed continuously throughout the reperfusion period. Bile output and hepatic clearance (expressed as percentage of BSP in bile samples) were determined at 120 min of reperfusion. ATP, adenine nucleotides, and lipid peroxidation were evaluated in the liver samples at 120 min of reperfusion. Light/electron microscopy analysis of the liver was also performed at 120 min of reperfusion.

Biochemical determinations

Transaminase assay Hepatic injury was evaluated according to transaminase levels using commercial kit from Boehringer Mannheim (Munich, Germany).

Nucleotide analysis Livers were homogenized in perchloric acid solution, and the adenine nucleotides pool was measured by high-performance liquid chromatography^[32].

Bile output Liver function was assessed by measuring bile production^[33,34]. Bile was collected through the cannulated bile duct and output reported as $\mu\text{L/g}$ liver.

Hepatic clearance As with bile output, hepatic clearance was considered as another parameter of hepatic function^[35,36]. Thirty minutes after the onset of the perfusion (t_{30}), 1 mg of BSP (Sigma, Spain) was added to the perfusate. The concentration of BSP in bile samples (t_{20}) was measured at 580 nm with an UV-visible spectrometer. Bile BSP excretion was expressed as a percentage of perfusate content (t_{20} bile/ t_{30} perfusate $\times 100$)^[35,36].

Flow rate and vascular resistance Liver circulation was assessed by measuring perfusion flow rate and vascular resistance^[34,37]. Perfusion flow rate was assessed continuously throughout the reperfusion period and expressed as mL/min g. Vascular resistance was defined as the ratio of portal venous pressure to flow rate and expressed in mmHg·min g/mL^[34,37].

Lipid peroxidation assay Lipid peroxidation was used as an indirect measure of the oxidative injury induced by ROS^[19,38]. Lipid peroxidation was determined by measuring

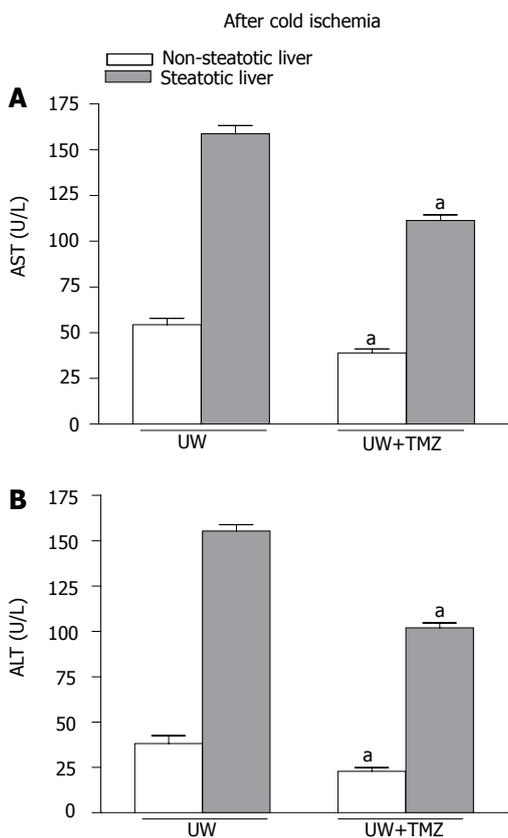


Figure 1 AST (A) and ALT (B) levels in flushing effluent after 24-h cold storage. UW: liver preserved in UW solution; UW+TMZ: liver preserved in UW with TMZ. ^a*P*<0.05 vs UW.

the formation of malondialdehyde (MDA) with the thiobarbiturate reaction^[19].

Light/electron microscopy

For light microscopy examinations, liver samples were fixed in 10% neutral buffered formalin and embedded in Paraplast, and 5-μm sections were stained with hematoxylin and eosin according to standard procedures^[19]. For electron microscopy, the fixation of hepatic tissue was performed using a 2.5% glutaraldehyde/2% paraformaldehyde. These samples were post-fixed with osmium tetroxide and potassium ferrocyanide, dehydrated in acetone, and embedded in Spurr's medium. Ultrathin sections were prepared with an ultracut-E ultramicrotome and contrasted with uranyl acetate and lead citrate. Stained sections were reviewed under an H 600-AB Hitachi electron microscope^[39].

Statistics

Data were expressed as mean±SE, and were compared statistically by variance analysis, followed by Student-Newman-Keuls test. *P*<0.05 was considered significant.

RESULTS

Protocol 1: Effect of TMZ on steatotic liver injury after 24 h of cold ischemia

Flushing of livers before reperfusion allowed effluent fluid to collect for the determination of transaminases^[6,40]. This

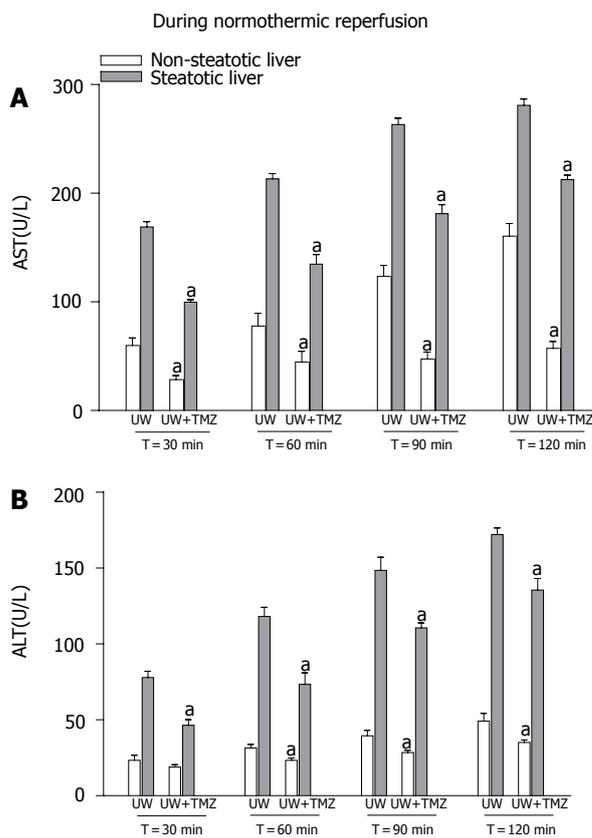


Figure 2 AST (A) and ALT (B) levels in perfusate after 30, 60, 90, and 120 min of normothermic reperfusion. UW: liver preserved in UW solution; UW+TMZ: liver preserved in UW with TMZ. ^a*P*<0.05 vs UW.

measure proved to be a valuable tool for predicting organ damage after cold preservation^[6,40]. As shown in Figure 1, the higher levels of transaminases released by steatotic livers after cold preservation in UW solution confirm the increased sensitivity of this type of liver to cold ischemia. The addition of TMZ to UW solution (UW+TMZ) reduced transaminase levels in the flushing effluent of non-steatotic livers and, in particular in that of steatotic livers.

Protocol 2: Effect of TMZ on steatotic liver injury after 24 h of cold ischemia followed by 2 h of normothermic reperfusion

Higher perfusate transaminase levels were observed in steatotic livers as the reperfusion period progressed compared with those found in non-steatotic livers (Figure 2). The addition of TMZ to UW solution reduced the perfusate transaminase release in both types of liver, but especially in steatotic livers. The histological light microscopic study showed a marked disintegration of hepatic architecture in both types of liver preserved in UW solution (Figures 3A and B), but especially in steatotic livers (Figure 3B), whereas in the UW+TMZ group hepatocyte integrity was maintained (Figures 3C and D).

Liver function was assessed by measuring bile production and BSP clearance. Bile output and the percentage of BSP in bile were lower in steatotic livers preserved in UW solution than in non-steatotic livers (Figure 4). Both liver function parameters improved significantly in the two liver types following the addition

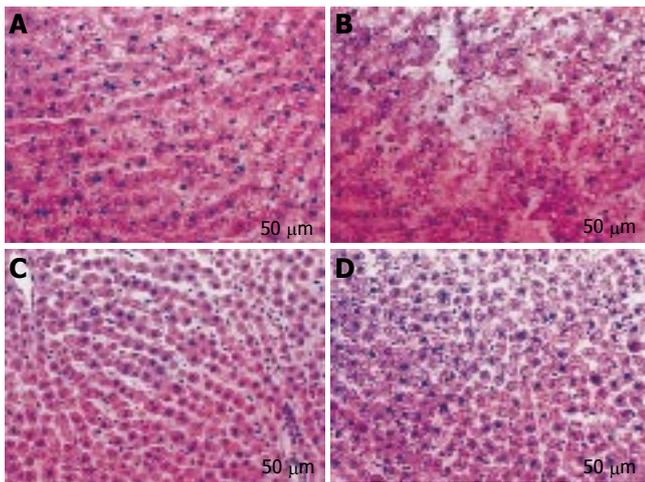


Figure 3 Histological change in steatotic and non-steatotic livers after 120 min of normothermic reperfusion. Non-steatotic (A) and steatotic (B) livers preserved in UW solution: Cell swelling and disintegration of hepatic architecture were more evident in steatotic livers. Non-steatotic (C) and steatotic (D) livers preserved in UW solution with TMZ: Hepatic morphology was better preserved in both types of the liver compared to that recorded in UW solution.

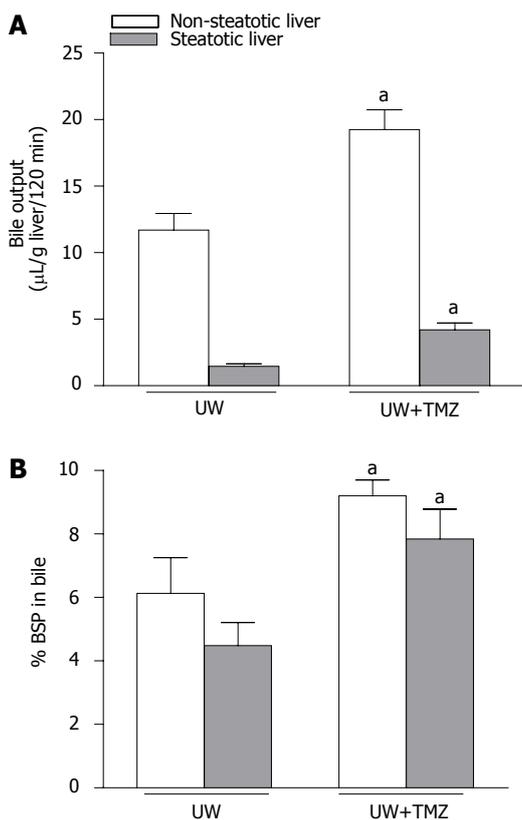


Figure 4 Bile output (A) and percentage of sulfobromophthalein (BSP) in bile (B) of steatotic and non-steatotic livers after 120 min of normothermic reperfusion. UW: livers were preserved in UW solution; UW+TMZ: liver preserved in UW with TMZ. ^a*P*<0.05 vs UW.

of TMZ to UW solution (UW+TMZ).

The mechanisms by which TMZ was able to protect steatotic livers against the deleterious effects of I/R injury were also evaluated. As shown in Figure 5, MDA levels during reperfusion increased, particularly in the case of steatotic livers. The addition of TMZ to UW

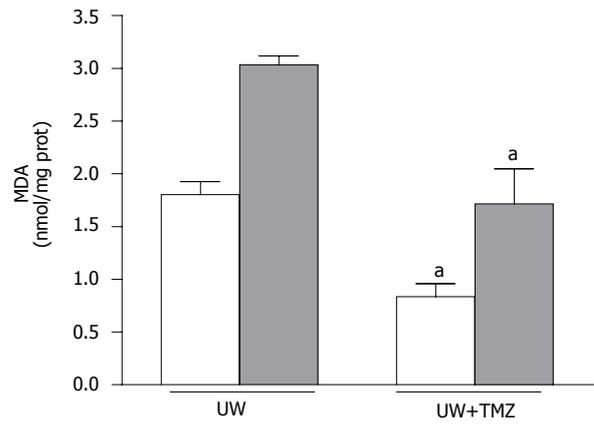


Figure 5 Hepatic malondialdehyde (MDA) levels after 120 min of normothermic reperfusion. UW: liver preserved in UW solution; UW+TMZ: liver preserved in UW with TMZ. ^a*P*<0.05 vs UW.

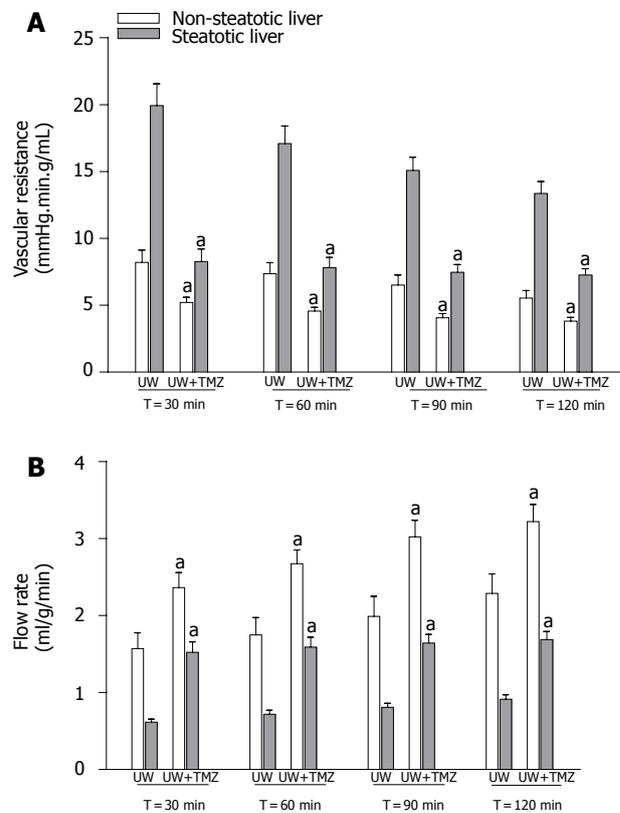


Figure 6 Vascular resistance (A) and perfusion flow rate (B) of steatotic and non-steatotic livers after 120 min of normothermic reperfusion. UW: livers were preserved in UW solution; UW+TMZ: liver preserved in UW with TMZ. ^a*P*<0.05 vs UW.

solution (UW+TMZ) brought about a reduction in these levels of increase. Marked increases in the vascular resistance and lower perfusion flow rate were observed in steatotic livers preserved in UW solution during the reperfusion period ($t_{30,60,90,120}$) compared with the rates in non-steatotic livers (Figure 6). The liver weight was not statistically significant in both liver types. Vascular resistance was lower and perfusion flow rate was higher in both types of liver following the addition of TMZ to UW preservation solution (UW+TMZ). The beneficial

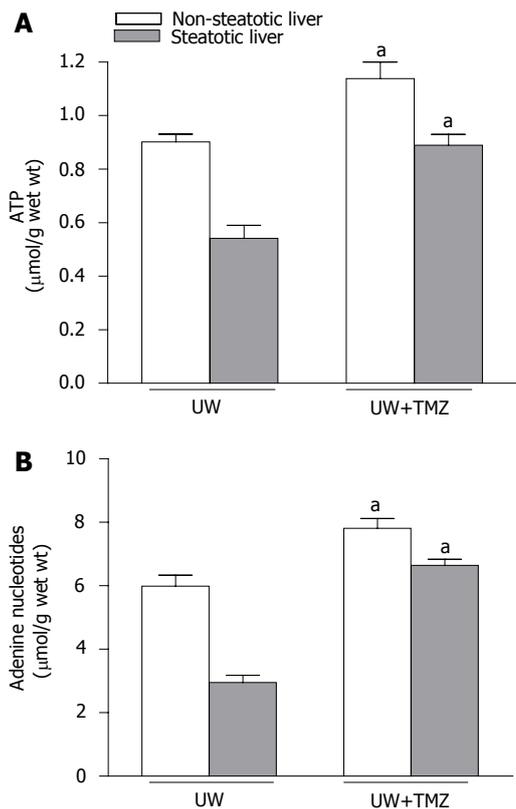


Figure 7 ATP (A) and adenine nucleotide (B) levels of steatotic and non-steatotic livers after 120 min of normothermic reperfusion. UW: livers were preserved in UW solution; UW+TMZ: liver preserved in UW with TMZ. ^a $P < 0.05$ vs UW.

effect of TMZ on the vascular resistance and perfusion flow rate were more evident in the steatotic livers (Figure 6). Lower ATP and adenine nucleotides levels during reperfusion were observed in steatotic livers preserved in UW solution than those recorded for non-steatotic livers (Figure 7). The results reported here show that TMZ led to the preservation of more ATP and adenine nucleotides content in both types of liver.

Mitochondrial damage was evaluated by electron microscopy. Ultra-structural analysis after reperfusion in both types of liver preserved in UW solution revealed swelling of mitochondria, a significant decrease in the electron density of their matrices, while cristae were only seldom visible (Figures 8A and B). These morphological alterations were more evident in steatotic livers (Figure 8B). Following the addition of TMZ to UW preservation (UW+TMZ) a greater degree of mitochondrial preservation in both types of liver was recorded (Figures 8C and D) compared to the results obtained for the livers in UW solution.

DISCUSSION

The results of the present study indicate that the addition of TMZ to UW solution improved the capacity of this standard preservation solution in both types of liver subjected to prolonged ischemic period especially in steatotic livers.

It is widely accepted that bile analysis is a useful

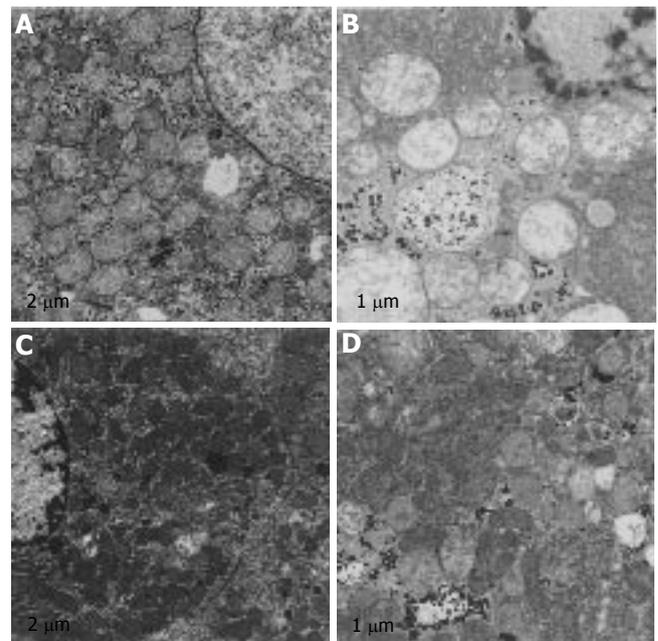


Figure 8 Electron microscopy analysis of structural integrity of the mitochondria. Non-steatotic (A) and steatotic (B) livers preserved in UW solution: mitochondria swelling and the alterations in the electron density of their matrices were more evident in steatotic livers. Cristae are only seldom visible in steatotic livers. Non-steatotic (C) and steatotic (D) livers preserved in UW solution with TMZ. Mitochondrial integrity was better preserved in both types of liver compared to that recorded in UW solution.

means for assessing the integrity of biliary epithelial cells after cold ischemia. If cold storage time exceeds 10-12 h, complications in biliary structures occur in more than 25% of liver transplant recipients^[41-43]. Several factors, including poor recovery after ATP depletion appear to contribute to bile duct cell damage after liver transplantation. Furthermore, isolated rat bile duct epithelial cells are noticeably sensitive to oxidative stress, possibly because their cellular stores of reduced glutathione are seven times lower than those of hepatocytes^[44]. Taking these observations into account, bile production failure in steatotic livers could be explained, at least partially, by the lower ATP and increased oxidative stress presented by this type of liver compared with non-steatotic liver. Liver transplantation may benefit from strategies such as the addition of TMZ to the preservation solution as this seems to help maintain appropriate bile duct cell functions. Here, this strategy increased ATP recovery and reduced oxidative stress. This was also associated with better bile production.

The lower perfusate flow rate and the higher resistance to flow in steatotic, as opposed to non-steatotic livers indicate that steatotic livers offer greater impediments to perfusion. Fat accumulation in the cytoplasm of the hepatocytes is associated with an increase in cell volume, which may result in the partial or complete obstruction of the hepatic sinusoidal space^[3,21]. Our results indicate the beneficial effects of TMZ on the flow rate and vascular resistance especially in the steatotic livers which were preserved during prolonged cold ischemia. Various hypotheses could be forwarded in an attempt at explaining the underlying protective mechanisms. In fact, according

to the literature, TMZ reduces the leakage of intracellular potassium, which is a vasoconstrictor; it affects vasoactive mediators including prostaglandins; and it reduces tissue edema, which has been related to the disturbance of microvascular circulation^[10,45,46].

Fatty degeneration, which induces a series of ultrastructural and biochemical alterations both in human and animal mitochondria^[17,47] may render these organelles intrinsically more susceptible to I/R injury. Given that mitochondria are the main sites for ATP and ROS production in I/R^[16], the lower ATP and adenine nucleotides content and the increased oxidative stress observed in steatotic livers preserved in UW solution could be attributed to mitochondrial damage. Thus, the identification of new strategies to prevent mitochondrial injury should represent an important research goal in attempts to optimize the use of donor steatotic organs for liver transplantation. Our result indicates that the addition of TMZ to UW preservation solution protects against mitochondrial damage caused by I/R, preserves more ATP and adenine nucleotide content during reperfusion and decreases oxidative stress, thus showing a protective effect against I/R injury. Taken together, these data suggest that mitochondria might be an important target through which TMZ exerts its cytoprotective effect. However, the underlying mechanisms are still far from being defined and other possibilities should not be ruled out. Thus, another possible reason for the higher ATP levels that are induced by TMZ might be improved microcirculation at the time of reperfusion. This could increase the availability of oxygen and, therefore, facilitate ATP production. It is well known that failure of liver perfusion can impair the ability of the steatotic liver to restore ATP levels^[16,22,48]. Similarly, in addition to mitochondria, the beneficial effects of TMZ on oxidative stress could be exerted on other sources of ROS, including endothelial cells^[10].

The addition of TMZ to UW solution ensured that steatotic grafts were less susceptible to the mechanisms involved in I/R injury including microcirculatory diseases, ATP and adenine content, oxidative stress and mitochondrial damage. In addition to allopurinol and glutathione, TMZ could be another antioxidant that should be added to UW solution, considering the benefits of TMZ in terms of oxidative stress and the difficulties for preventing oxidative stress damage in steatotic liver by other pharmacological treatments^[19,33,49]. The ability of TMZ to protect both types of the liver is particularly attractive since some strategies that are effective in non-steatotic livers may not be useful in the presence of steatosis^[19,20,50].

In conclusion, TMZ protected against hepatic injury and ameliorated liver function in steatotic and non-steatotic livers preserved in UW solution. Further studies will be required to elucidate whether TMZ is such a promising drug for the field of liver transplantation, and if this pharmacological substance can reduce the inherent risk of steatotic liver grafts for transplantation.

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Epidural anaesthesia restores pancreatic microcirculation and decreases the severity of acute pancreatitis

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Abstract

AIM: To investigate the effect of epidural anaesthesia (EA) on pancreatic microcirculation during acute pancreatitis (AP).

METHODS: AP was induced by injection of sodium taurocholate into the pancreatic duct of Sprague-Dawley rats. To realize EA, a catheter was introduced into the epidural space between T7 and T9 and bupivacaine was injected. Microcirculatory flow was measured by laser Doppler flowmetry. Arterial blood gas analyses were performed. At the end of the experiment (≤ 5 h), pancreas was removed for histology. The animals were divided into three groups: Group 1 ($n=9$), AP without EA; Group 2 ($n=4$), EA without AP; and Group 3 ($n=6$), AP treated by EA.

RESULTS: In Group 1, pancreatic microcirculatory flow prior to AP was 141 ± 39 perfusion units (PU). After AP, microcirculatory flow obviously decreased to 9 ± 6 PU ($P < 0.05$). Metabolic acidosis developed with base excess (BE) of -14 ± 3 mmol/L. Histology revealed extensive edema and tissue necrosis. In Group 2, EA did not significantly modify microcirculatory flow. BE remained unchanged and histological analysis showed normal pancreatic tissue. In Group 3, AP initially caused a significant decrease in microcirculatory flow from 155 ± 25 to 11 ± 7 PU ($P < 0.05$). After initiation of EA, microcirculatory

flow obviously increased again to 81 ± 31 PU ($P < 0.05$). BE was -6 ± 4 mmol/L, which was significantly different compared to Group 1 ($P < 0.05$). Furthermore, histology revealed less extensive edema and necrosis in pancreatic tissue in Group 3 than that in Group 1.

CONCLUSION: AP caused dramatic microcirculatory changes within the pancreas, with development of metabolic acidosis and tissue necrosis. EA allowed partial restoration of microcirculatory flow and prevented development of tissue necrosis and systemic complications. Therefore, EA should be considered as therapeutic option to prevent evolution from edematous to necrotic AP.

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Key words: Acute pancreatitis; Epidural anaesthesia; Pancreatic blood flow; Microcirculation; Taurocholic acid

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INTRODUCTION

Among patients suffering from acute pancreatitis (AP), 80% have a favorable evolution and approximately 20% develop a severe disease with significant morbidity and mortality^[1]. Severe AP is associated with the development of local complications, such as pancreatic and peripancreatic necrosis, abscesses or pseudocysts, and systemic complications, such as adult respiratory distress syndrome or renal failure with a mortality close to 15%^[2].

The pathophysiology of AP is incompletely understood but alteration in the pancreatic microcirculatory blood flow has been involved. Thus, a decrease in pancreatic blood flow occurs early in the course of AP and has been suggested to play a role in the conversion of edematous to necrotizing AP^[3-7]. The microcirculatory dysfunction includes arterial vasoconstriction with hypoperfusion, ischemia-reperfusion injury, and obstruction of the venous outflow^[8-11]. Other factors that participate in the development of microcirculatory

dysfunction are hemoconcentration, hypercoagulability, and increase in microvascular permeability^[8-11]. Besides perfusion abnormalities, AP is also characterized by local and systemic inflammatory responses, including leukocyte activation as well as release of free radicals and cytokines^[12-14].

Many therapeutic agents, such as dextran, heparin, procaine, L-arginine, antioxidants, or cytokine antagonists, have been tested experimentally and/or clinically to improve pancreatic tissue perfusion during AP, however, no significantly successful result has been achieved^[1,4,15-18].

Epidural anesthesia (EA) that is used to induce analgesia in the perioperative period might be an interesting treatment of the microcirculatory blood flow abnormalities. Thus, EA can reduce the incidence of post-operative pulmonary complications, and shorten the duration of the post-operative intestinal paralysis^[19,20]. In addition, experimental studies have shown that EA increases gut mucosal blood flow and delays the metabolic acidosis due to intestinal ischemia in the presence of a decreased perfusion pressure^[21-22]. The beneficial effect of EA has been attributed to a sympathetic nerve blockade, which redistributes blood flow to the non-perfused regions of the gut^[21]. Additionally, EA is useful to decrease pain in patients with AP^[23], but no study has investigated the effects of EA on the pancreatic microcirculatory blood flow during AP.

Because we postulated that EA might improve the pancreatic hypoperfusion induced by AP and concomitantly decrease the severity of the disease, we measured the pancreatic microcirculatory blood flow and the severity of the disease in rats injected with taurocholic acid in the biliopancreatic duct, in the presence or in the absence of EA.

MATERIALS AND METHODS

Animals

Adult male Wistar rats (275-300 g) were provided by Charles River (L'Arbresle, France). Animals were bred and housed in standard cages and maintained in climate-controlled rooms. Animals were fed with standard laboratory chow, given water *ad libitum*, and randomly assigned to control or treated groups. The protocol was approved by the Animal Ethical Committee of the Geneva University Medical School and by the Geneva Veterinary Authorities.

Epidural anaesthesia

Epidural catheters were placed in rats under isoflurane anesthesia. A polyethylene catheter (PE10, Portex, Kent, UK) was introduced in the lumbar region and positioned into the epidural region between T7 and T9 according to the technique previously described by Grouls *et al*^[24]. The external end of the catheter was tunneled subcutaneously and fixed to the occiput.

The animals were allowed to recover from anesthesia and 1 h after epidural catheterization, bupivacaine (4 g/L, 20 μ L) was injected to test anaesthesia. Hind limb muscle tone was scored by manual inspection and visual observation as previously described during 30 min^[21,24]. A

normal tone with free movement of the hind limbs was scored 0; weak hypotonia of the hind limbs and of the body was scored 1; moderate hypotonia of the hind limbs and of the body was scored 2; and inability to support the body on the hind limbs and flat body posture was scored 3. After scoring, rats were allowed to recover for 2 h. Animals showing signs of neurologic damage were discarded. To verify the position of the catheter, after rat sacrifice, Evans blue solution was injected in the catheter and the spinal column exposed. Animals that had the catheter tip located intrathecally or outside the region between T7 - T9 were excluded from the study.

Surgical preparation

Anaesthesia was induced by pentobarbital sodium (50 mg/kg intraperitoneally) and isoflurane inhalation. Rats had tracheotomy under isoflurane anesthesia and were mechanically ventilated (Harvard apparatus, model 683, South Natick, MA) with a Fio₂ = 0.5. The left femoral vein was cannulated and continuously perfused with saline solution (2.5 mL/100 g/h). The left femoral artery was cannulated for continuous monitoring of arterial blood pressure and blood gas analysis (ABL 505 Analyzer, Radiometer, Copenhagen, Denmark). Body temperature was kept constant with a warm pad.

Blood samples were harvested every 30 min for blood gas analysis and ventilation was adjusted to obtain pCO₂ between 35 and 45 mmHg. The blood samples were also analyzed for serum amylase concentrations using 4,6-ethylidene (G1)-p-nitrophenyl (G1)- α D-maltoheptoside (Sigma Chemical Co, Zurich, Switzerland) as substrate. Mean arterial pressure was continuously recorded and stored via an analog-digital interface converter (Biopac, Santa Barbara, CA) on an AST microcomputer (AST, Limerick, Ireland).

Induction of acute pancreatitis

After laparotomy, the pancreatic duct was cannulated with a 22-gauge catheter (Abott, Sligo, Ireland)^[25]. A clip was placed on the pancreatic duct close to the liver and taurocholic acid (5%, 500 μ L, Sigma, Saint-Louis, MO) was infused over 4 min with a micropump^[25].

Pancreatic microcirculatory blood flow

Pancreatic microcirculatory blood flow was measured with a laser Doppler flowmetry (Periflux system 5000, Perimed AB, Järfälla, Sweden). To position the probe, a latex adhesive probe miniholder (model PH07, Perimed, Järfälla, Sweden) and a special articulated laboratory stand were used. The Doppler probe was placed under the pancreatic surface with the light directed upward. After a 10 to 15 min stabilization period, the effect of AP and/or EA on the pancreatic microcirculatory blood flow was measured over time. The pancreatic microcirculatory blood flow was continuously recorded and stored via an analog-digital interface converter (Biopac, Santa Barbara, CA) on an AST microcomputer (AST, Limerick, Ireland).

Tissue samples

After the rats were sacrificed, pancreatic samples were rapidly collected, fixed in formalin, embedded in paraffin,

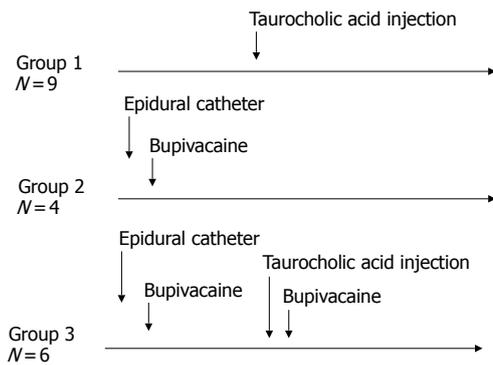


Figure 1 Experimental design. Acute pancreatitis was induced in Groups 1 and 3 by injection of taurocholic acid (5%, 500 μ L) into the biliopancreatic duct. Epidural anaesthesia was induced in Groups 2 and 3 by epidural injection of bupivacaine (4 g/L, 20 μ L).

and cut into 5- μ m thick sections. After staining with hematoxylin-eosin, the sections were examined by two experienced morphologists who were not aware of the sample identity. The extent of acinar cell necrosis was quantified by computer assisted morphometry as previously described^[26] and expressed as a percent of total acinar tissue.

Experimental design

Three experimental groups were studied (Figure 1). In Group 1 ($n=9$), rats had AP induction and no EA. Pancreatic microcirculatory blood flow was measured before taurocholic acid injection and continued after the induction of AP. In Group 2 ($n=4$), the pancreatic microcirculatory blood flow was determined before and continued after the induction of EA. In Group 3 ($n=6$), 30 min after taurocholic acid injection, bupivacaine was injected via the epidural catheter and the pancreatic microcirculatory blood flow measured over time.

Statistical analysis

Results were expressed as mean \pm SD. Nonparametric Wilcoxon Signed ranks test and Kruskal-Wallis tests for comparison between groups were used as appropriate. $P<0.05$ was considered statistically significant.

RESULTS

Epidural anaesthesia in rats

EA was tested by bupivacaine injection in Groups 2 and 3. In all rats, hind limb blockade was complete within 2 min and the motility completely recovered within 25–30 min after the injection. In Group 3, rats had taurocholic acid injection to induce AP and were treated with bupivacaine (Figure 1).

Pancreatic microcirculatory blood flow

In Group 1, induction of AP caused a significant decrease of mean pancreatic microcirculatory blood flow from 141 ± 40 units to 9 ± 6 units (96%, $P=0.008$) within 30 min (Figure 2). The decreased blood flow remained unchanged until the end of the experiment. In Group 2, EA slightly increased microcirculatory blood flow, but the

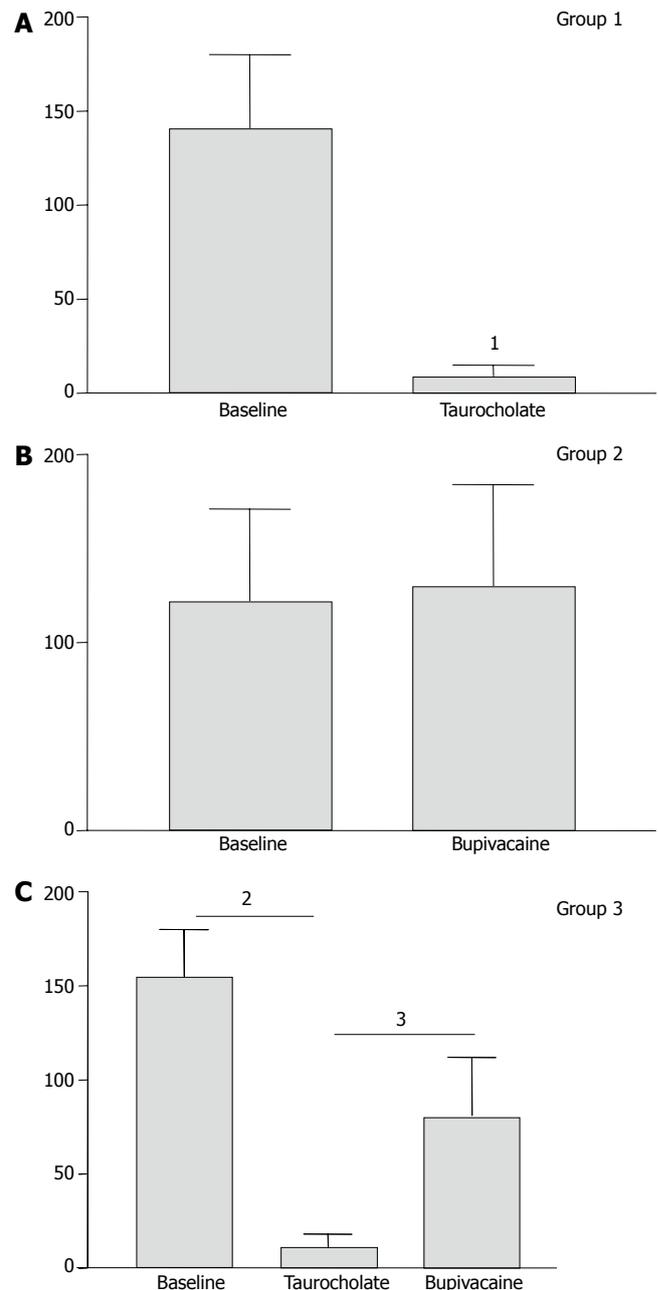


Figure 2 Pancreatic microcirculatory blood flow (arbitrary units). In Group 1, the mean value of pancreatic blood flow measured prior to and after induction of AP showed a significant decrease of perfusion from 141 ± 40 units to 9 ± 6 units (96%) ($P=0.008$) within 30 min. In Group 2, the mean value of pancreatic blood flow measured prior to and after induction of epidural anaesthesia showed no significant modification of perfusion. In Group 3, mean value of pancreatic blood flow measured prior to and after induction of AP, and epidural anaesthesia, respectively. Induction of AP caused a significant decrease in pancreatic microcirculatory flow from 155 ± 25 units to 11 ± 7 units (93%) ($P=0.004$). After induction of epidural anaesthesia, mean pancreatic microcirculatory blood flow increased again significantly to 81 ± 31 units within 45 min, reaching 52% of baseline values ($P=0.028$).

modification did not reach statistical significance ($P=0.7$, Figure 2). In Group 3, induction of AP caused a similar decrease in pancreatic microcirculatory flow as in Group 1, *i.e.*, from 155 ± 25 units to 11 ± 7 units (93%, $P=0.004$). After induction of EA, mean pancreatic microcirculatory blood flow increased again significantly to 81 ± 31 units within 45 min, reaching 52% of baseline values ($P=0.028$, Figures 2 and 3).

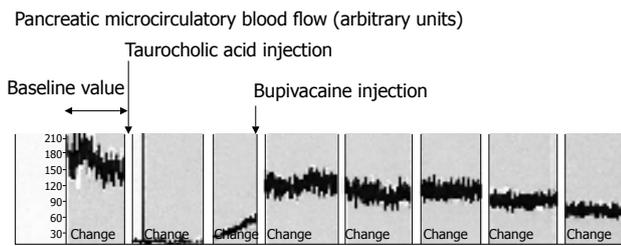


Figure 3 Recording of pancreatic microcirculatory blood flow in Group 3. Rats were injected with taurocholic acid and treated 30 min later with bupivacaine (0.4%, 20 μ L) in the epidural catheter. Induction of AP caused a significant decrease in pancreatic microcirculatory blood flow. After induction of epidural anesthesia, mean pancreatic microcirculatory blood flow increased again significantly within 45 min, reaching approximately 50% of baseline values.

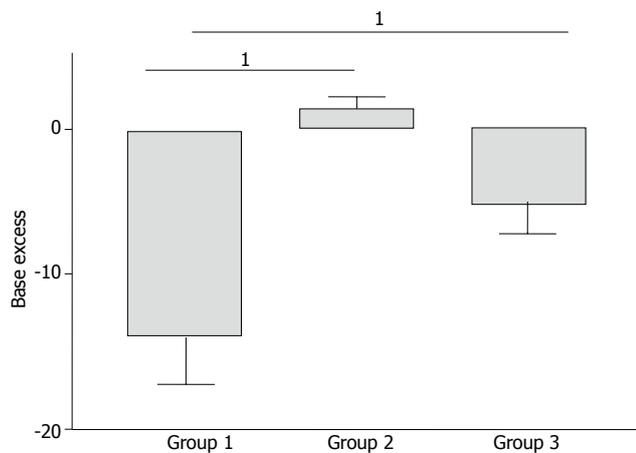


Figure 4 Arterial blood gas analyses. In Group 1, severe metabolic acidosis developed progressively reaching a maximum base excess (BE) value of -14 ± 3 mmol/L at the end of the experiment. In Group 2, BE values remained unchanged compared to baseline values. In Group 3, a mild metabolic acidosis developed reaching a maximum BE value of -6 ± 4 mmol/L at the end of the experiment. This result was significantly improved compared to Group 1 ($^1P=0.007$).

Arterial blood gas and serum amylase analyses

In Group 1, severe metabolic acidosis developed progressively reaching a maximum base excess (BE) value of -14 ± 3 mmol / L at the end of the experiment (Figure 4). In Group 2, BE values remained unchanged compared to the baseline values during experiment (Figure 4). In Group 3, a mild metabolic acidosis developed reaching a maximum BE value of -6 ± 4 mmol/L at the end of the experiment (Figure 4). This result was significantly improved compared to Group 1 ($P=0.007$).

In Group 1, serum amylase levels increased from 694 ± 419 units/L prior to AP to 2178 ± 561 units/L ($P < 0.05$) after 2 h of induction of AP (Figure 5). In Group 2, EA did not modify serum amylase level significantly during the experiment. In Group 3, serum amylase levels increased to a maximum of 1829 ± 641 units/L 2 h after initiation of AP and EA (Figure 5). Although this result was lower compared to Group 1, but did not reach statistical significance.

Histopathology

In Group 1, histopathology revealed a severe form of AP with extensive edema and tissue necrosis (Figure 6C). In

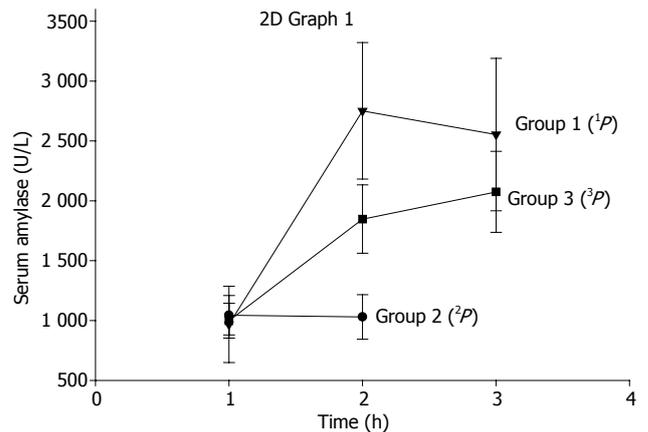


Figure 5 Serum amylase levels. In Group 1, serum amylase levels increased from 694 ± 419 units/L prior to AP to 2178 ± 561 units/L ($^1P < 0.05$, vs serum amylase levels before induction of AP) after 2 h of induction of AP. In Group 2, epidural anaesthesia did not modify serum amylase levels significantly ($P=0.9$). In Group 3, serum amylase levels increased to a maximum of 1829 ± 641 units/L after 2 h of initiation of AP and epidural anesthesia. Although this result was lower compared to Group 1, but did not reach statistical significance ($^2P=0.08$).

Group 2, almost normal pancreatic tissue was observed at the end of the experiment (Figure 6B). In Group 3, edema and necrosis were less extensive compared to animals in Group 1 (Figure 6D).

The extent of acinar cell necrosis measured as percentage of total surface at high power field (HPF) showed that Group 2 animals had similar cell necrosis as the controls ($< 10\%$) (Figure 7). Group 1 animals demonstrated over 40% of acinar cell necrosis at HPF, whereas acinar cell necrosis was below 30% in Group 3 animals (Figure 7).

DISCUSSION

In our study, taurocholic acid injection in the biliopancreatic duct induced a pancreatic hypoperfusion as reported by previous studies^[3,7,16,25]. The decrease was -96% within 15 min and pancreatic microcirculatory blood flow remained low until the end of the experiment. The microcirculatory blood flow has been accurately measured by laser Doppler flowmetry. The technique is ideal for monitoring changes in tissue perfusion over time and has successfully been applied to numerous organs in experimental and human studies^[4,6,24,27]. However, several factors, such as artifacts induced by respiration, external light exposure and temperature, can affect the interpretation of the laser Doppler signals^[4]. In addition, microcirculatory flow can vary widely over short distances, and small changes in probe angle during measurement can alter the measured values. To prevent these technical problems, we used a new type of probe holder, which allows the tissue to be maintained by gravity, avoiding repeated repositioning of the probe.

More importantly, we showed that after induction of AP, bupivacaine injection in the epidural catheter increased pancreatic microcirculatory blood flow. Concomitantly, the improved microcirculatory blood flow within the pancreas decreased the severity of AP. Serum amylase

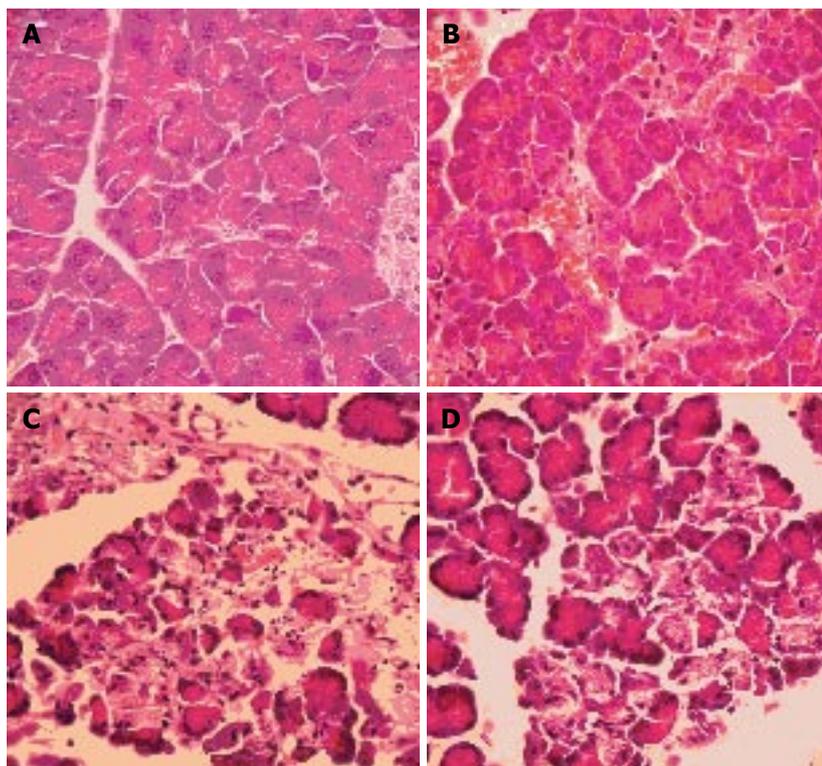


Figure 6 Histopathological features of pancreatic tissue. **A:** A control animal was sacrificed and histopathology showed normal pancreas; **B:** Group 2 animals showing normal pancreatic tissue with minor modifications, possibly related to vasodilatation and surgical manipulations; **C:** Group 1 animals showing a severe form of AP with extensive edema and tissue necrosis; and **D:** Group 3 animals demonstrating less extensive pancreatic edema and necrosis compared to Group 1 animals.

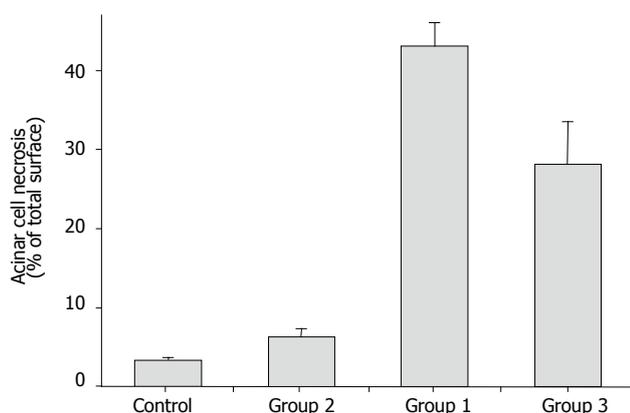


Figure 7 Acinar cell necrosis (percentage of total pancreatic surface). The measure of acinar cell necrosis by percentage of total surface at high power field (HPF) showed that Group 2 animals had similar cell necrosis as the controls (<10%). Group 1 animals demonstrated over 40% acinar cell necrosis at HPF, whereas acinar cell necrosis was below 30% in Group 3 animals.

concentrations and metabolic acidosis were less severe and the histopathologic scores were improved in comparison to rats with AP and no treatment. These results emphasize the importance of a decreased microcirculatory blood flow in inducing severe AP.

To our best of knowledge, this is the first experimental study showing the beneficial effects of EA on the severity of AP. Previous reports already showed that EA increased splanchnic venous capacitance and decreased arterial tone by blocking sympathetic nerve activity^[21,22,28-30]. It was also reported that EA might increase sympathetic activity and vasoconstriction in organs distant from the anesthetized area and redistributes blood flow towards splanchnic organs^[21].

It has been postulated that several factors, such as local metabolic acidosis which activates various proteases, oxygen-free radical that injures endothelium and parenchyma, or the incapacity of plasma protease inhibitors to circulate through acinar cells, participate in the modifications of pancreatic microcirculation during AP^[3]. These modifications result in diminished intravascular volume, chemically-induced vasoconstriction, intravascular coagulation, and increased endothelial permeability^[31]. Finally, pancreatic ischemia, as a consequence of all these local effects, may convert a mild disease to a severe AP with parenchymal necrosis^[31]. This has been demonstrated by Klar *et al*^[5] who have shown in anesthetized rabbits that pancreatic blood flow increases when AP is edematous (cerulein injection), whereas pancreatic blood flow decreases when AP is severe (necrotizing form induced by taurocholate injection).

In our study, EA decreased serum amylase concentrations, metabolic acidosis, and the severity of pancreatic necrosis score in comparison to rats that had similar taurocholic acid injection and no treatment. Similar benefit of peridural anaesthesia has been shown on metabolic acidosis during hypoxia in dogs^[29].

In conclusion, the current study has shown that EA improves the pancreatic hypoperfusion induced by AP with a concomitant decrease in the severity of metabolic acidosis and a diminished tissue injury. EA should therefore be considered a new therapeutic approach to prevent the progression from an edematous disease to a necrotizing AP.

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Expression, purification and characterization of enterovirus-71 virus-like particles

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Abstract

AIM: Enterovirus 71 (EV71) has been implicated as the etiological agent responsible for the recent outbreaks of hand, foot and mouth disease associated with severe neurological diseases in the Asia-Pacific region.

METHODS: The assembly process was hypothesized to occur via an orchestrated proteolytic processing of the P1 precursor by the viral protease 3CD. To test this hypothesis, we constructed 3 recombinant baculoviruses: Bac-P1 expressing P1; Bac-3CD expressing 3CD; and Bac-P1-3CD co-expressing P1 and 3CD.

RESULTS: Both single infection by Bac-P1-3CD and co-infection by Bac-P1 and Bac-3CD resulted in correct cleavage of P1 to yield individual proteins VP0, VP1 and VP3, while the former approach yielded higher VLP production. In the cells, the structural proteins self-assembled into clusters of virus-like particles (VLP) resembling the authentic EV71 particle aggregates. After ultracentrifugation purification, the dispersed VLPs were indistinguishable from the authentic virus in size, appearance, composition and surface epitopes, as determined by SDS-PAGE, Western blot, transmission electron microscopy and immunogold labeling.

CONCLUSION: Our data, for the first time, suggest that in insect cells EV71 structural proteins adopt a processing and assembly pathway similar to poliovirus assembly. The preservation of particle morphology and composition suggest that the VLP may be a valuable vaccine candidate to prevent EV71 epidemics.

INTRODUCTION

Enterovirus 71 (EV71), like poliovirus, is one of the 69 enterovirus serotypes belonging to the *Picornaviridae* family. EV71 infection manifests most frequently as hand, foot and mouth disease (HFMD) and children under 5 years of age are particularly susceptible to the most severe forms of EV71-associated neurological disease, including aseptic meningitis, brainstem encephalitis and acute flaccid paralysis indistinguishable from poliomyelitis^[1]. The neurovirulence of EV71 first came to people's attention in 1975 in Bulgaria when 44 people died of a polio-like disease^[2]. EV71 was further implicated as the causative agent responsible for the epidemics of central nervous system disease occurring in New York, Europe, Australia and Asia^[3-6]. Recent HFMD outbreaks also affected thousands of children in Taiwan^[7], Japan^[8] and Singapore^[9], causing the fatality of more than 80 children. Thus EV71 appears to emerge as an increasingly important neurotropic enterovirus in the upcoming era of poliomyelitis eradication. However, no effective antiviral drugs or vaccines are available yet.

EV71 possesses a positive single-stranded RNA genome that consists of a single open reading frame (ORF). The ORF expresses a large polyprotein that can be co- and post-translationally cleaved into P1, P2 and P3 regions^[10]. P1 region encodes the four structural proteins VP1, VP2, VP3 and VP4, while P2 and P3 regions encode other nonstructural proteins (e.g., 2A and 3CD) responsible for virus replication and virulence^[11]. Based on a model derived from poliovirus, protease 2A autocatalytically cleaves P1 at its N-terminus and liberates P1 from the nascent polyprotein^[11], while protease 3CD cleaves P1 precursor into VP1, VP3 and VP0 in trans^[12]. These three structural proteins spontaneously assemble into icosahedral

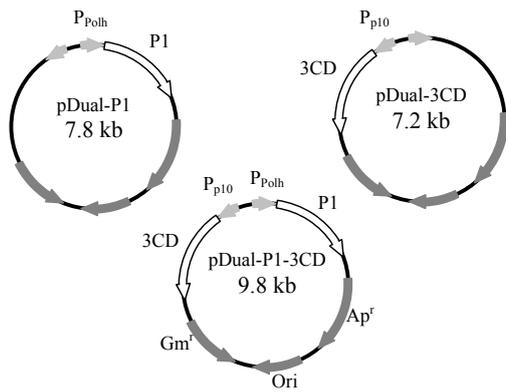


Figure 1 Construction of three recombinant baculovirus vectors using pFastBac™ DUAL plasmid as the backbone. The gene fragments encoding P1 and 3CD were cloned separately into MCS I and II of pFastBac™ DUAL under the control of polyhedrin (P_{polh}) and p10 promoters (P_{p10}), respectively. The resultant plasmids were designated pDual-P1 and pDual-3CD, respectively. The P1 and 3CD genes were cloned together into MCS I and II under the control of polyhedrin and p10 promoters. The resultant plasmid was designated pDual-P1-3CD.

procapsid in an ordered manner and proceed through a series of intermediates, followed by the encapsidation of the RNA genome into the provirion^[13]. The final encapsidation step involves the cleavage of VP0 into VP2 and VP4, therefore the final mature virion consists of 60 copies each of VP1 and VP3, 58-59 copies of VP2 and VP4 and 2-1 copies of VP0^[13].

Besides the mature virions, naturally occurring virus-like particles (VLPs) of poliovirus have been discovered^[14]. VLPs are empty particles consisting of viral structural proteins but devoid of viral nucleic acids, hence they are non-infectious. VLPs can generally induce broad and strong immune responses thanks to the preservation of many essential epitopes^[15-17], therefore VLPs have captured increasing attention as potential vaccine candidates^[17-19]. To date, numerous VLPs of clinically important viruses, including human immunodeficiency virus^[20], SARS coronavirus^[21] and hepatitis delta virus^[22], have been expressed. In addition, recombinant poliovirus VLPs have been produced using the baculovirus/insect cell or vaccinia virus expression system. These particles were produced via the (1) expression of the complete ORF; (2) co-expression of individual VP0, VP1 and VP3 proteins; or (3) co-expression of P1 and 3CD proteins^[23-25]. Since EV71 and poliovirus share identical genome organization and similar protein functions, we hypothesized that EV71 assembly occurs in a way similar to poliovirus and have previously demonstrated the formation of EV71 VLP by co-infecting insect cells with two recombinant baculoviruses, Bac-P1 expressing P1 and Bac-3CD expressing 3CD^[26]. However, the factors influencing the VLP yield were not investigated, and the VLPs were not purified or characterized. In this study, we extended our previous work by further constructing a new recombinant baculovirus simultaneously expressing P1 and 3CD. The new virus was used for single infection of insect cells, co-expression of P1 and 3CD and synthesis of VLP. The yields of VLP produced via the single infection and co-infection strategies were compared. Finally, the VLPs were purified and characterized.

MATERIALS AND METHODS

Cell culture and media

Spodoptera frugiperda (Sf-9) insect cells were cultured in spinner flasks (Bellco) at 27 °C using TNM-FH medium supplemented with 100 mL/L fetal bovine serum (FBS, Gibco BRL) as described elsewhere^[27].

Generation of recombinant baculoviruses

The gene fragments coding for P1 and 3CD were amplified by polymerase chain reaction from the full-length cDNA clone of EV71 *neu* strain^[28] and subcloned separately into multiple cloning site (MCS) I and II of pFastBac™ DUAL plasmids (Invitrogen) as described previously^[26]. The resultant plasmids were designated pDual-P1 and pDual-3CD, respectively. Likewise, the P1 gene fragment was cloned into MCS I under the polyhedrin promoter and the 3CD gene fragment was inserted into MCS II under the p10 promoter. The resultant plasmid carrying two gene cassettes was designated pDual-P1-3CD (Figure 1). The recombinant baculoviruses were subsequently generated using Bac-to-Bac® system (Invitrogen) as described previously^[29] and designated Bac-P1, Bac-3CD and Bac-P1-3CD based on the genes they encoded. The recombinant viruses were propagated, amplified, stored and titered according to standard procedures^[30].

SDS-PAGE and Western blot

To confirm the expression of recombinant P1 and VP1, the infected cells were harvested by low-speed centrifugation and then lysed by incubation in the lysis buffer (0.1% Triton X-100 in Tris-buffered saline) at 4 °C for 30 min. The proteins were resolved on 100 g/L gels as described previously^[26] and stained by Coomassie blue. For Western blot, the proteins were transferred onto nitrocellulose membranes according to standard procedures (Bio-Rad Laboratories). The transferred membrane was blocked with 50 g/L non-fat milk and probed by mouse anti-VP1 monoclonal antibody (MAb). The secondary antibody was goat anti-mouse IgG conjugated with alkaline phosphatase (Kirkegaard and Perry Laboratories). The membranes were developed with BCIP/NBT color developing reagent (Sigma).

Purification of VLP by ultracentrifugation

EV71 VLPs were produced by infecting Sf-9 cells with Bac-P1-3CD at a multiplicity of infection (MOI) of 10. Approximately 150 mL cells (1.5×10^6 cells/mL) were harvested at 2 d post-infection (dpi) by centrifugation (1 000 r/min for 5 min) and were re-suspended in 10 mL TE buffer (0.01 mol/L Tris-HCl, 0.001 mol/L EDTA, pH 7.4). The cell suspension was supplemented with 1 mL of 10X HO buffer (0.01 mol/L Tris, 0.25 mol/L NaCl, 0.01 mol/L 2-mercaptoethanol) and 100 g/L NP-40, lysed by sonication for 3 min and centrifuged at 13 000 g (R20A2 rotor, CR22G, Hitachi) for 30 min. The supernatant was passed through a 0.22- μ m filter to remove the debris and then ultracentrifuged at 27 000 g (P40ST rotor, CP100MX, Hitachi) for 4 h. The pellets were resuspended in TE buffer, sonicated for 30 s and loaded onto the discontinuous sucrose gradient (15%, 30% and 65%

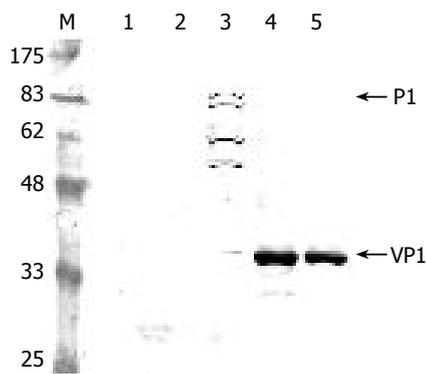


Figure 2 Western blot analysis of cell lysates infected by different viruses. The Sf-9 cells were infected by the viruses at a total MOI of 10 and harvested at 3 dpi. The proteins were separated by SDS-PAGE, electrotransferred to a nitrocellulose membrane and probed using anti-VP1 MAb as the primary antibody. Lane 1: mock infection; Lane 2: wild-type baculovirus AcMNPV; Lane 3: Bac-P1; Lane 4: Bac-P1-3CD; Lane 5: Bac-P1 and Bac-3CD.

sucrose dissolved in TE buffer). After ultracentrifugation at 27 000 *g* (P40ST rotor) for 3 h, the milky white band between the interfaces of 15–35% sucrose was collected and layered on top of a 1.33 *g cm*⁻³ cesium chloride (CsCl) solution. After ultracentrifugation at 34 000 *g* (P40ST rotor) for 24 h, the bands collected from CsCl gradient were dialyzed against phosphate-buffered saline (pH 7.4) and were ultracentrifuged again at 34 000 *g* for 5 h. The pellets were resuspended in 50 μ L pure water for SDS-PAGE, Western blot and transmission electron microscopy (TEM) analyses. The purities of the samples and the composition of VLPs were quantified by densitometry using Scion Image Shareware (Scion Corporation).

TEM and immunogold labeling

The VLPs within the insect cells were visualized under a transmission electron microscope (H-7500, Hitachi), prior to which ultrathin sectioning of cells was performed as described previously^[26]. The particles after purification were adsorbed onto Formvar-coated copper grids, negatively stained by 20 *g/L* phosphotungstic acid (PTA) and examined by TEM. For immunogold labeling, purified particles were adhered onto copper grids and probed by mouse anti-VP1 MAb. The secondary antibody was goat anti-mouse IgG conjugated with 5 nm gold particles (Sigma). After washing of the unbound gold particles, the grids were stained by 20 *g/L* PTA and observed by TEM.

RESULTS

Expression of recombinant proteins in insect cells

To determine whether the recombinant P1 and 3CD were successfully expressed by Bac-P1-3CD, Sf-9 cells were singly infected by wild-type AcMNPV, Bac-P1, and Bac-P1-3CD (MOI 10), or co-infected by Bac-P1 (MOI 5) and Bac-3CD (MOI 5). The cells were harvested at 3 dpi and lysed for Western blot analysis. As shown in Figure 2, the mock-infected and wild-type AcMNPV-infected cells did not express P1 (lanes 1 and 2), whereas the Bac-P1-infected cells expressed a protein with a molecular mass corresponding to 97 ku, indicating the

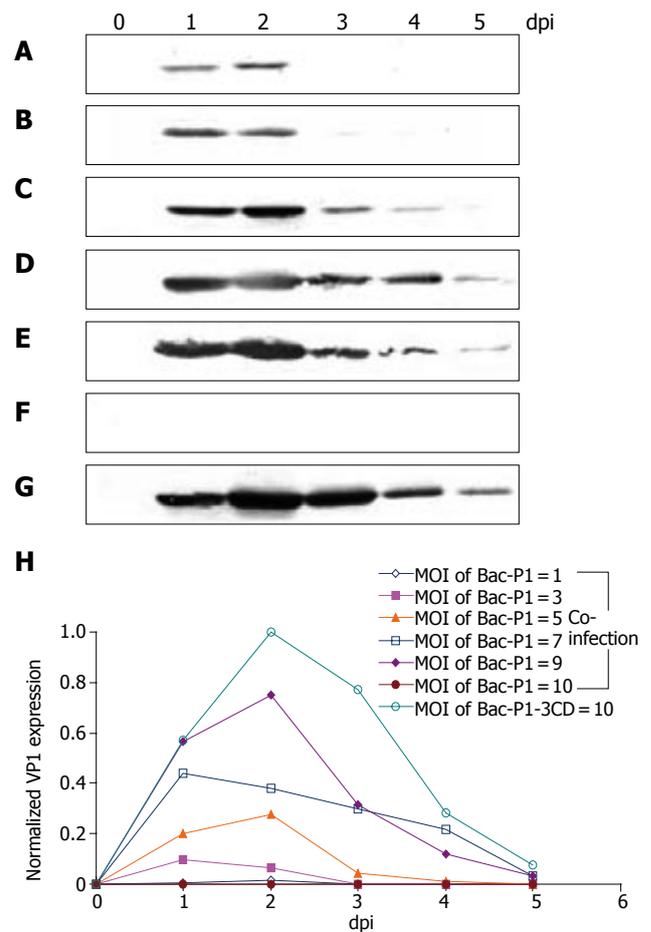


Figure 3 Time course profiles of VP1 production under various infection conditions as analyzed by Western blot. Insect cells were infected at a total MOI of 10 by single infection with Bac-P1-3CD (G), or co-infection at MOI ratios (Bac-P1/Bac-3CD) of 1/9 (b), 3/7 (B), 5/5 (C), 7/3 (D), 9/1 (E) and 10/0 (F). Lanes 0–5 represent the cell lysates collected at 0, 1, 2, 3, 4, and 5 dpi, respectively. The relative VP1 production levels under different conditions were quantified by scanning densitometry (H). The data represent the average values of duplicated experiments.

successful expression of P1 (lane 3). Other P1-associated proteins migrating faster than P1 were also found (lane 3), suggesting a possible degradation of P1 by the cellular proteases, but this non-specific degradation did not yield VP1 (39 ku).

We also noted that no antibody specifically recognizing 3CD was available, hence the 3CD expression was detected indirectly by observing whether P1 was cleaved into VP1 upon co-expression of both proteins. Lanes 4 and 5 depict that either single infection by Bac-P1-3CD (lane 4) or co-infection by Bac-P1 and Bac-3CD (lane 5) led to complete cleavage of P1 into VP1, proving the successful co-expression of functional P1 and 3CD by both approaches.

Optimization of VP1 production

Since P1 relies on 3CD for cleavage and subsequent VLP assembly, delicately controlling the relative expression levels of P1 and 3CD is crucial for optimal VLP production. To address this issue, the cells were co-infected by Bac-P1 and Bac-3CD at MOI ratios of 1/9, 3/7, 5/5, 7/3, 9/1 and 10/0, or singly infected by Bac-P1-3CD at MOI 10 in order to compare and optimize the VP1 yield. Figures (3A–3G) show the time-course profiles

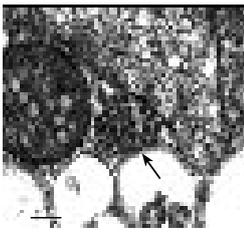


Figure 4 Electron microscopy examinations of EV71 VLP aggregates formed in cytoplasm of the Bac-P1-3CD-infected insect cells (100 000 X magnification). The cells were infected at MOI 10, harvested at 3 dpi and ultrathin sectioned for TEM. The arrowhead indicates the VLP aggregate. Bar:100 nm.

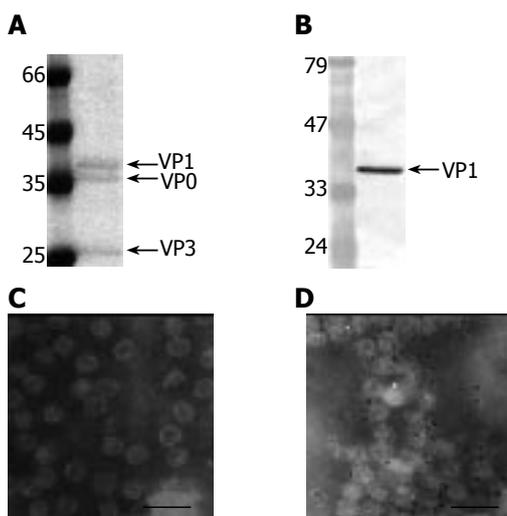


Figure 5 Purification and characterization of EV71 VLP (100 000 X magnification.). The VLPs were produced by infecting 150 mL Sf-9 cells (1.5×10^6 cells/mL) with Bac-P1-3CD at MOI 10, harvested at 2 dpi and purified by ultracentrifugation. The purified VLPs were characterized by SDS-PAGE (A), Western blot (B), TEM examination (C) and immunogold labeling (D). The PAGE gel was stained by Commassie blue and scanned for densitometry analysis. The primary antibody used in the Western blot and immunogold labeling was anti-VP1 MAb. The size of the gold particles was 5 nm. Bar: 100 nm.

of VP1 production, in which lanes 0-5 represent the samples harvested at 0, 1, 2, 3, 4 and 5 dpi, respectively. As shown, both single infection and co-infection approaches resulted in similar expression kinetics (except MOI 10/0), that is, the VP1 concentration rapidly culminated at 1-2 dpi, declined thereafter and virtually vanished at 5 dpi. The concentration peaked slightly earlier compared to those of many other proteins whose maximum concentration occurred at 3 dpi^[27,31] probably because of proteolytic degradation by cellular proteases (data not shown).

The production levels using different MOI combinations were also compared by scanning densitometry, by which all band intensities were normalized against the strongest band intensity (lane 2, Figure 5G). Figure 5H reveals that in the case of co-infection VP1 concentration increased with the MOI of Bac-P1 provided that the total MOI was fixed at 10. However, the maximum VP1 yield obtained from single infection (by Bac-P1-3CD at MOI 10) was the highest, being $\approx 30\%$ and $\approx 120\%$ greater than those obtained from co-infection at MOI 9/1 and 7/3, respectively.

Formation of intracellular virus-like particle aggregates

Since single infection with Bac-P1-3CD yielded the highest VP1 production, the formation of VLPs by single infection strategy was further examined. The cells were singly infected by Bac-P1-3CD at MOI 10 and subjected to ultrathin sectioning and TEM examination. As shown in Figure 4, clusters of virus-like particles were observed in the cytoplasm. These particles measured 25-27 nm in diameter and appeared icosahedral, thus resembling the authentic enterovirus in size and appearance. Such VLP aggregates were not observed in Sf-9 cells infected by Bac-P1 alone (data not shown), indicating that the particles were not the assembly products of individual structural proteins degraded from P1 precursor.

Purification and characterization of EV 71 VLP

The subsequent VLP production was performed by infecting insect cells with Bac-P1-3CD (MOI 10). The particles were released from the infected cells, purified by ultracentrifugation and analyzed by SDS-PAGE (Figure 5A) and Western blot (Figure 5B). We observed that the purified sample contained three major proteins whose molecular masses corresponded to those of VP1 (39 ku), VP0 (36 ku) and VP3 (26 ku), and the purity of the sample was greater than 90% (Figure 5A). The molar concentrations of the three proteins as determined by scanning densitometry were roughly equal, agreeing with the notion that the molar ratios of VP0, VP1 and VP3 are identical in the enterovirus provirions^[13]. Because no antibodies specifically recognizing VP0 and VP3 were available, the Western blot was performed using anti-VP1 antibody only, which further confirmed that the 39 ku protein was VP1. The purified particles were also subjected to TEM examination (Figure 5C), which showed that particles with size and morphology similar to those of the particles observed *in vivo* except the purified VLPs were dispersed. The immunogold labeling using anti-VP1 antibody further revealed gold particles surrounding the distinct particles (Figure 5D), indicating the exposure of VP1 on the outer surface. All these data confirmed that the Bac-P1-3CD infection successfully resulted in the formation of VLP comprising VP0, VP1 and VP3 and these particles could be purified by ultracentrifugation to near homogeneity.

The purified VLPs were also used as the standard to measure the specific yields of VLP. Approximately 10 mg of VLP was expressed per 10^9 Bac-P1-3CD-infected cells at MOI 10. This high expression level may contribute to future mass production and downstream purification.

DISCUSSION

The assembly pathway of EV71 has not been definitely identified, but the assembly model of poliovirus suggests that concerted cleavage of P1 by protease 3CD is required for capsid assembly. In this study, 3 recombinant baculoviruses were employed to express P1 and 3CD simultaneously in order to mimic the natural assembly process. By either single infection with Bac-P1-3CD or co-infection with Pac-P1 and Bac-3CD, successful processing

of P1 into VP1 (Figure 1) was demonstrated, proving the successful expression of both P1 and functional 3CD. Alternative approach by co-expression of individual VP1, VP2, VP3 and VP4 proteins has been tested for VLP assembly, yet the yield was very low (personal communication). The comparison of these approaches suggests that VLP synthesis from the P1 precursor is a more favorable route, which agrees with the assembly pathway of poliovirus VLP^[24].

The co-infection experiments further suggested that the amount of P1 required was larger than that of 3CD for VLP assembly as the co-infection at MOI 9/1 yielded considerably larger amounts of VP1 than MOI 5/5 or 1/9 (Figure 3). Intriguingly, we observed that single infection by Bac-P1-3CD was superior to co-infection by Bac-P1 and Bac-3CD with regard to VP1 production (Figure 3H). Since we lacked proper antibodies to detect 3CD, the relative expression levels of P1 and 3CD in the cells singly infected by Bac-P1-3CD remained unclear. However, since P1 expression was driven by polyhedrin promoter which was stronger than p10 promoter regulating the 3CD expression, one may surmise that differential expression of P1 and 3CD arose, namely, the P1 expression exceeded the 3CD expression. Another reason that may account for the higher production by single infection methodology is the VLP-producing cell population distribution as a result of the co-infection approach, whereby only the cells that were co-infected could produce VLP. Using the co-infection methodology, the probability of a cell to be infected by one or two types of viruses and the number of virus entering any given cells follow the modified Poisson distribution as predicted previously^[32]. Hence, a population of cells that were singly infected by Bac-P1 or Bac-3CD arose, and no P1 processing or VLP assembly occurred in these cells. Even for the cells that were co-infected by both viruses, the number of infecting Bac-P1 and Bac-3CD within one cell might vary^[32]. In cells where the number of Bac-P1 infection far more exceeded that of Bac-3CD infection, insufficient expression of 3CD and inadequate P1 cleavage could occur. On the contrary, in cells where the Bac-3CD infection considerably outnumbered the Bac-P1 infection, a drought in P1 supply could occur and lead to inadequate VLP assembly. Such heterogeneity could decrease the P1 processing and VLP assembly in many cells. In contrast, the infection of Bac-P1-3CD alone resulted in P1 processing in all cells, leading to a higher VP1 yield.

Despite the lower yield, the significance of co-infection strategy should not be ignored because it offers a new dimension to manipulate the composition of VLPs. For instance, the cells may be co-infected with Bac-P1-3CD and a new recombinant baculovirus expressing VP1 derived from a different EV71 strain (e.g., the prototype BrCr strain). The phenotypic mixing may allow the incorporation of VP1 from BrCr strain into the VLP, thus generating chimeric, multivalent VLPs and broadening the spectrum of protection.

In addition to the higher expression level, single infection strategy resulted in the formation of VLP morphologically indistinguishable from the authentic viruses. Within the cells, clusters of virus-like particles

resembling those observed in EV71-infected human rhabdomyosarcoma (RD) cells^[26] and in enterovirus-infected skeletal muscle cells^[33] were observed. The *in vivo* formation of particle aggregates was not previously reported by other researchers studying the poliovirus VLPs^[23,25], but was recently noted in BHK-38 cells infected by foot-and-mouth disease virus^[34], a relatively genetically distant picornavirus. To date, the biological significance of the particle aggregation remains to be explored, but the resemblance in particle morphology and the aggregation patterns between the VLP and the authentic enterovirus suggests that EV71 VLP adopts an assembly pathway in insect cells in a way similar to the authentic viruses do in their natural host cells. Despite the aggregation within cells, the VLP could be dispersed in solution and purified through CsCl gradient ultracentrifugation to near homogeneity. The purified VLPs comprised VP0, VP3 and VP1 of roughly equal molar ratios, a result consistent with previous findings that poliovirus provirions are composed of 60 copies each of VP1, VP3 and VP0^[13]. This implied the similarity between the VLP and the authentic virus in composition. Notably, in insect cells VP0 did not appear to undergo further cleavage into VP2 and VP4, a maturation process associated with the acquisition of infectivity and the increase in particle stability^[35]. The lack of further processing is reasonable as the natural maturation process takes place after the RNA encapsidation, yet in this expression system, no RNA packaging into the central cavity occurred.

Nonetheless, after purification, the size and morphology of VLPs appeared indistinguishable from those of poliovirus VLP. More importantly, the immunogold labeling confirmed the presence of VP1 epitopes on the VLP surface. These data suggest that the VLPs contain the important antigenic sites essential for eliciting the immune responses. This is particularly important because neutralizing epitopes are most densely clustered on VP1 and many of them consist of amino acids from different domains or from different polypeptides^[36,37], that is, many of them are conformation-dependent. In this regard, the use of VLP as a vaccine candidate against EV71 infection may be particularly promising. In recent years, VLP-based vaccines have been demonstrated to not only elicit neutralizing antibodies, but also provoke potent cytotoxic and helper T-lymphocyte responses and have been shown to confer protection from virus challenge in the animal models^[38-40]. Furthermore, papillomavirus-like particles have been shown to induce acute activation of dendritic cells^[16]. Other VLPs that have been evaluated in pre-clinical studies for their potentials as vaccines include JC virus, human immunodeficiency virus, rotavirus and parvovirus^[41]. Moreover, the humoral, mucosal and cellular immune responses to Norwalk VLP in humans have been reported^[42], while VLP vaccines against human papillomavirus have entered into phase II clinical trials and promising data have been presented^[43]. All these further justify the development of VLP vaccines.

Since EV71 has caused seasonal morbidity and mortality in children with increasing frequency in recent years, vaccine development has become a top priority among all control strategies. Although other vaccine types,

such as inactivated virus and VP1 subunit vaccines, have been tested^[44], only the inactivated virus elicited strong immune responses, again confirming the importance of preserving epitope conformation. However, further assessment of safety and efficacy of the inactivated virus vaccine is required. In this study, we have successfully demonstrated the formation and purification of EV71 VLP via co-expression of P1 and 3CD and a reasonable production yield (≈ 10 mg/ 10^9 cells) was achieved, thus offering an attractive alternative to the inactivated virus vaccine. At present, the immunological evaluation has been impeded due to the difficulty in obtaining sufficient amounts of pure VLP because the recovery yield of VLP by ultracentrifugation was very low. The poor recovery yield may be attributed to the impaired VLP stability as empty capsids are less stable than mature virions^[45]. The particle stability may be enhanced by the addition of a stabilizer (e.g., pirodavir, disoxaril or R 78206) that binds the pocket underneath the canyon of the particle^[46]. The feasibility of enhancing VLP integrity by stabilizers is being evaluated. Furthermore, the development of a more efficient chromatography-based purification scheme is necessary for process scale-up and is underway.

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CLINICAL RESEARCH

Cyclooxygenase-2 and epithelial growth factor receptor up-regulation during progression of Barrett's esophagus to adenocarcinoma

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INTRODUCTION

Esophageal adenocarcinoma (EAC) is one of the most rapidly increasing cancers over the past two decades in Caucasian males in the United States^[1,2]. After being diagnosed with adenocarcinoma, patients have only a 13-15% 5-year survival rate^[3]. Most of the EAC are developed from Barrett's esophagus (BE), a pre-malignant disease characterized by replacement of the normal esophageal squamous epithelium with a specialized intestinal metaplasia (SIM)^[4]. The risk of EAC in BE patients has been estimated to be about 30 -125 times higher than the general population^[2,5].

The mechanisms of the evolution from BE to EAC are largely unknown^[6,7]. During the neoplastic transformation, aberrations in the cell cycle lead to uncontrolled cellular replication, a molecular mechanism is believed to be involved in all carcinogenesis^[8]. Epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases lies at the head of a complex signal transduction cascade that modulates cell proliferation, differentiation, adhesion and migration^[9]. EGFR is a transmembrane receptor composed of an extracellular ligand-binding domain and a cytoplasmic region with enzymatic activity. This structure enables signals to be transmitted across the plasma membrane activating gene expression and inducing cellular responses, such as proliferation and differentiation. Over-expression of EGFR has been shown to occur in various malignancies, including esophageal adenocarcinoma^[10].

Another important enzyme involved in tumor growth is cyclooxygenase-2 (COX-2)^[11]. There are two isoforms of COX enzyme. COX-1 is constitutively expressed in most tissues; however, COX-2 has a markedly different expression pattern. Normally, COX-2 is undetectable in most tissues, but can be expressed at high levels after induction with a variety of substances, including inflammatory mediators and mitogens^[12-14]. Epidemiological studies have shown that intake of aspirin, a nonsteroidal anti-inflammatory drug (NSAID) known as COX enzymes blocker, is associated with an up to 90% decreased risk of developing esophageal

Abstract

AIM: To investigate the expression of cyclooxygenase-2 (COX-2) and epithelial growth factor receptor (EGFR) throughout the progression of Barrett's esophagus (BE).

METHODS: COX-2 and EGFR protein expressions were detected by using immunohistochemical method. A detailed cytomorphological changes were determined. Areas of COX-2 and EGFR expression were quantified by using computer Imaging System.

RESULTS: The expressions of both COX-2 and EGFR increased along with the progression from BE to esophagus adenocarcinoma (EAC). A positive correlation was found between COX-2 expression and EGFR expression.

CONCLUSION: COX-2 and EGFR may be cooperative in the stepwise progression from BE to EAC, thereby leading to carcinogenesis.

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Key words: Cyclooxygenase -2 (COX-2); Epithelial growth factor receptor (EGFR); Barrett's esophagus; Carcinogenesis

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cancer^[15,16]. Experimental studies also have indicated that over-expression of COX-2 is associated with decreased apoptosis and cell-cell adhesion, and with increased proliferation and differentiation^[17,18].

Recently, experimental investigations have suggested a close relationship between COX-2 and EGFR. Expression of EGFR was markedly increased in the COX-2-transfected cells, and inhibition of COX-2 could suppress the induction of EGFR in these cells^[19]. It has also been demonstrated that activation of EGFR can induce COX-2 expression and prostaglandin production^[20]. Furthermore, a combination of an EGFR inhibitor with a nonselective COX-1/COX-2 inhibitor was shown to prevent the development of intestinal cancer in nude mice^[21]. Taken together, these data link COX and EGFR, and provide a theoretical basis whereby the COX-2 and EGFR signaling pathway may have additive effects of tumor growth. Some studies on expression of COX-2^[18] and EGFR^[22] in the human Barrett's esophagus specimens have been reported. However, to our knowledge, a detailed morphologic description of the esophageal epithelium expressing both COX-2 and EGFR in the BE metaplasia to dysplasia to adenocarcinoma sequence has not yet been performed.

In the present study, we therefore investigated the expressions of both COX-2 and EGFR in biopsy tissues from different histologic stages of BE by using immunohistochemical assay, and addressed the morphological features of Barrett's epithelium with over-expressed protein levels of COX-2 and EGFR in the histologic stages of metaplasia and dysplasia. Furthermore, we set forth to determine the correlation between COX-2 and EGFR in progression from the metaplasia to dysplasia to adenocarcinoma sequence.

MATERIALS AND METHODS

Case selection and review

The study samples were collected retrospectively from 104 patients who had undergone esophageal biopsy between 1996 and 2004. All of these patients were recruited from the Barrett's Esophagus registry at the University of Louisville with established diagnoses of SIM, indefinite/low-grade dysplasia (I/LGD), high-grade dysplasia (HGD), and EAC. No preoperative radio- or chemotherapy had been performed. None of the patients had received NSAIDs therapy. A microscope examination confirmed the diagnoses of SIM, LGD, HGD and EAC on these esophageal tissues reviewed by two pathologists independently, blinded to the subject's clinical history. Alcian-blue staining was used to identify any presence of SIM. Hematoxylin-eosin staining was used to identify any presence of dysplasia and EAC. A control group consisted of 30 subjects with nondetectable Barrett's (NB), defined by endoscopic findings of columnar-lined esophagus (CLE), but no pathologic evidence of SIM despite alcian-blue staining on all of the four-quadrant biopsies. This study was approved by the Institutional Review Board for Human Study at the University of Louisville.

Immunohistochemical assay for COX-2 and EGFR

COX-2 and EGFR protein expressions were determined

by using an immunohistochemical assay. Staining was carried out on the paraffin-embedded tissues using the DAKO EnVision™+System Kit (DAKO Corporation, Carpinteria, CA) according to the manufacturer's instructions. In brief, the sections were deparaffinized and hydrated. The slides were washed with TRIS-buffer, and peroxidase blocking was performed for 5 min. After rewashing, the slides were incubated separately with the monoclonal mouse COX-2 antibody (1:200) or EGFR antibody (1:200) (SantaCruz Biotechnology Inc, CA) for 30 min at room temperature. The chromogenic substrate diaminobenzidine was added as a visualization reagent. Finally, the slides were counterstained with haematoxylin. A negative control was included in each run.

Cytomorphology associated with localization of COX-2 and EGFR

Microscopic evaluation of the morphological features of epithelium was performed at high-power fields (X 400 magnification) on the COX-2 and EGFR staining sections. Two cytomorphological features were chosen as the criteria of assessment based on observed morphological differences among SIM, I/LGD, HGD and EAC along with NB columnar epithelium: (1) ordering of nuclei; and (2) the presence of basally located nuclei with an apical cytoplasmic layer. We considered the normal cell phenotype (NCP) when the epithelial cells were arranged in a single layer with their long axes orientated towards the center of the lumen, and had a basally located nucleus with a thick apical layer of cytoplasm, whereas the abnormal cell phenotype (ACP) included multiple cell layers, displayed an enlarged nucleus and less cytoplasm. Every slide was examined throughout to identify the NCP cell and ACP cell with COX-2 staining and EGFR staining. Then, the COX-2-stained and the EGFR-stained epithelial cells were categorized to NCP cells and ACP cells. The other cells were ignored during the evaluation.

Computer image analysis

A computer image analysis was performed to quantify the expressions of COX-2 and EGFR in totally 104 subjects diagnosed with SIM, I/LGD, HGD and EAC, and in 30 controls. The imaging fields were chosen randomly from various section levels to ensure objectivity of sampling. Five imaging fields were scanned for each specimen sample. All digital images were acquired with the microscope at 40x magnification using the Spot camera via the MetaMorph® Imaging System (Universal Imaging Corporation., Downingtown, PA) and stored as JPG data files (the resolutions were fixed as 200 pixels/inch). The acquired color images from the immunohistochemical staining were defined a standard threshold according to the software specification. The computer program then quantified the threshold area represented by color images. COX-2 and EGFR protein expressions were defined by the percentages of threshold area in acquired color images.

Statistical analysis

Spearman rank correlation coefficient (R) was used to analyze the correlation between COX-2 and EGFR expressions with the various histologic stages of Barrett's

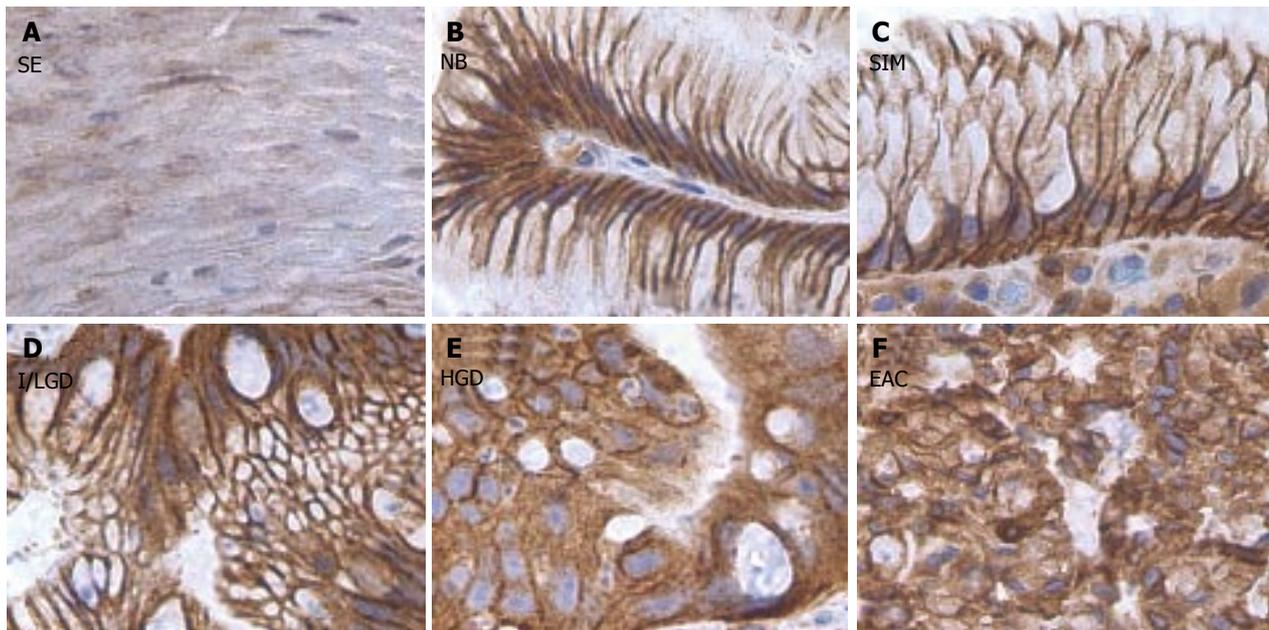


Figure 1 COX-2 staining in the sections from different stages of BE along with the sections from normal squamous and CLE nondetectable BE. **A:** Squamous epithelium (SE); **B:** Nondetectable BE in CLE (NB); **C:** Specialized intestinal metaplasia (SIM); **D:** Indeterminate/low-grade dysplasia (I/LGD); **E:** high-grade dysplasia (HGD); **F:** Esophageal adenocarcinoma (EAC). Strong COX-2 staining was detected in both NCP cells and ACP cells in the histological stages of SIM, I/LGD, HGD and EAC. A weak COX-2 staining was observed in the normal squamous epithelium.

Table 1 Demographics of 104 study subjects with Barrett's esophagus and 30 control subjects

| | NB | SIM | I/LDG | HDG | EAC |
|----------------------|------|------|-------|------|------|
| Subjects | 30 | 37 | 36 | 16 | 15 |
| Gender (male:female) | 28:2 | 34:3 | 30:6 | 14:2 | 14:1 |
| Mean age (yr) | 60 | 55 | 61 | 68 | 66 |
| Short-segment CLE | 27 | 21 | 7 | 1 | 0 |
| Long-segment CLE | 3 | 16 | 29 | 15 | 15 |

SIM: Specialized intestinal metaplasia; I/LGD: Indeterminate/low-grade dysplasia; HGD: High-grade dysplasia; EAC: Esophageal adenocarcinoma; NB: Nondetectable Barrett's; CLE: Columnar-lined esophagus.

esophagus. Multiple analysis of variance (MANOVA) was used to determine the differences between COX-2 and EGFR expressions, if any, between the different histologic stages of carcinogenesis. *P* values less than 0.05 were considered statistically significant.

RESULTS

One hundred and four subjects (92 males, 12 females) with Barrett's esophagus were enrolled in this study. The study population consisted of 37 subjects with a diagnosis of SIM without dysplasia, 36 with I/LGD, 16 with HGD, and 15 with EAC. The control group consisted of 30 NB subjects. Patients identified as having no Barrett's were mostly those with a short segment of CLE whereas those identified with SIM, I/LDG, HDG or EAC were mostly found to have a long segment of CLE (Table 1).

Cytomorphological features associated with localization of COX-2 and EGFR

A very strong COX-2 staining in all Barrett's epithelium compared to a weak staining in normal squamous epithelium and CLE without Barrett's epithelium was observed. In particular, in the regions with COX-2-positive staining, the epithelial cells presented cytomorphological features of both NCP cells and ACP cells in the histological stages of SIM, I/LGD, HGD and EAC (Figure 1). A positive EGFR staining was presented in all Barrett's epithelium, but not as strong as that seen in the COX-2 staining. In contrast to COX-2 staining, the regions of positive EGFR expression exhibited ACP cells while those regions with weak or no EGFR staining exhibited NCP cells (Figure 2).

Expression of COX-2 in the metaplasia to dysplasia to adenocarcinoma sequence

We observed significant increases in COX-2 expression in specimens with SIM (7.8 ± 0.8), I/LGD (10.0 ± 0.9), HGD (14.2 ± 1.8) and EAC (16.0 ± 2.2) as compared with the control specimens (6.9 ± 1) ($P < 0.05$). Furthermore, COX-2 expression appeared to increase with the histological severity of BE; there was a significant positive correlation between COX-2 expression and histopathologic changes from I/LGD to EAC ($r = 0.97$, $P < 0.001$) (Figure 3).

Expression of EGFR in the metaplasia-dysplasia-adenocarcinoma sequence

We found significant increases in EGFR expression in specimens with SIM (2.1 ± 0.5), I/LGD (2.7 ± 0.6), HGD (3.9 ± 0.7) and EAC (5.2 ± 0.9) as compared with the control specimens (0.8 ± 0.4) ($P < 0.05$). Furthermore, EGFR expression appeared to increase with the histological severity of BE; there was a significant positive

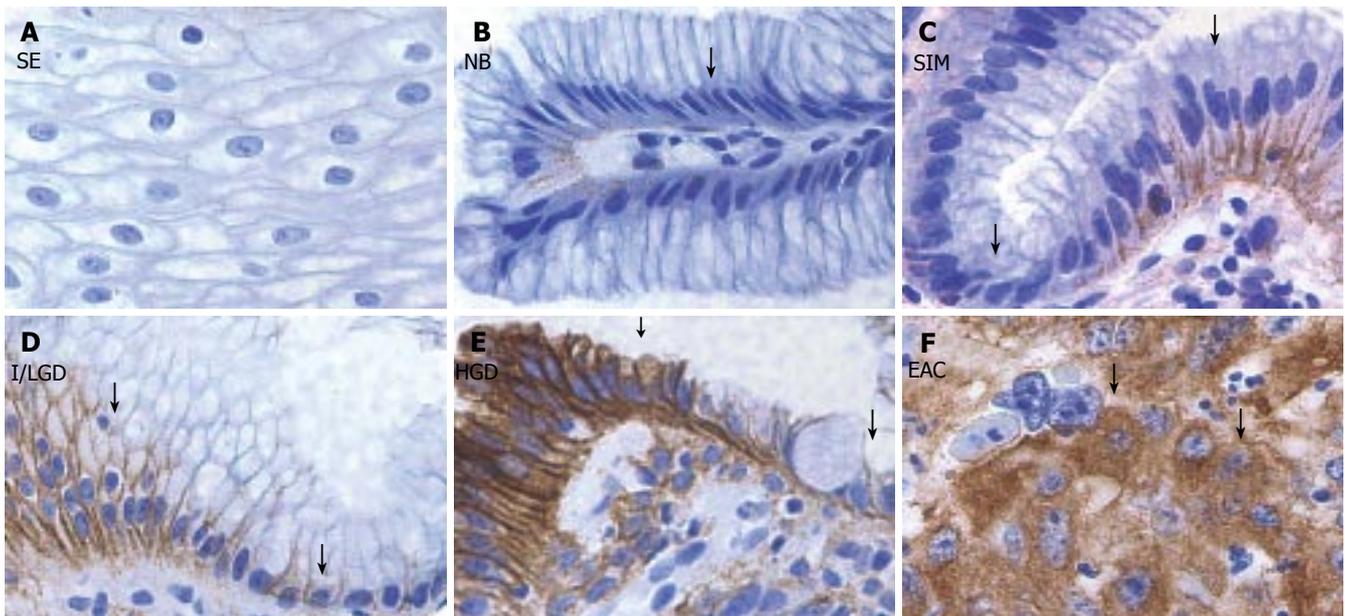


Figure 2 EGFR staining in the sections from different stages of BE along with the sections from normal squamous and CLE nondetectable BE. **A:** Squamous epithelium (SE); **B:** Nondetectable BE in CLE (NB); **C:** Specialized intestinal metaplasia (SIM); **D:** Indeterminate/low-grade dysplasia (I/LGD); **E:** high-grade dysplasia (HGD); **F:** Esophageal adenocarcinoma (EAC). Strong EGFR staining was detected in ACP cells (arrowhead) in the histological stages of SIM, I/LGD, HGD and EAC. A weak EGFR staining was found in NCP cells (arrow) and in the normal squamous epithelium.

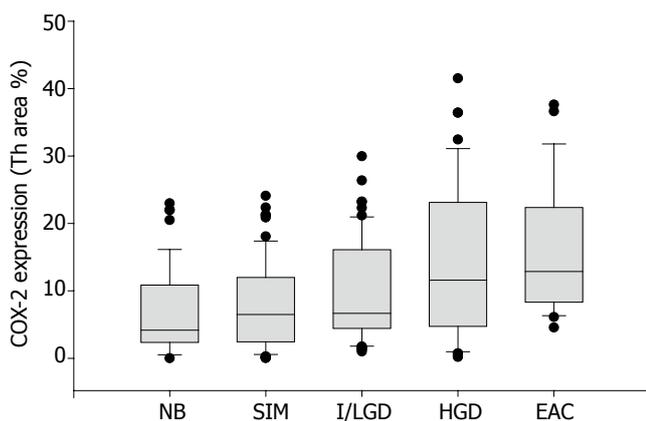


Figure 3 COX-2 expression and pathological severity of BE. COX-2 expression is plotted against the 4 stages of Barrett's esophagus. The line enclosed within the box represents the median value; the small circles indicate outlying data points. COX-2 expression in specimens with SIM, I/LGD, HGD and EAC was significantly increased in comparison with that in NB ($P < 0.01$). A significant positive correlation between COX-2 expression and histopathologic changes from SIM to EAC was observed ($r = 0.97$, $P < 0.001$).

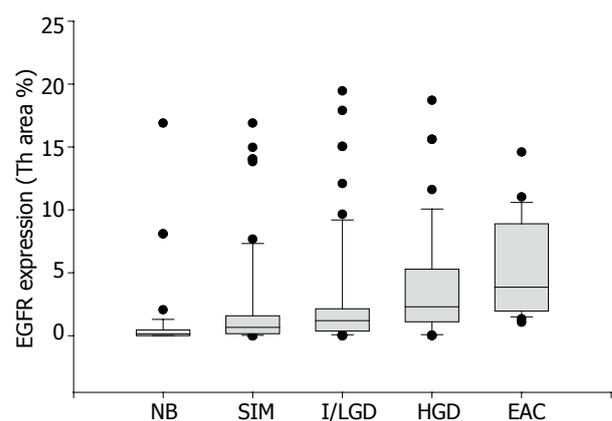


Figure 4 EGFR expression and pathological severity of BE. EGFR expression is plotted against the 4 stages of Barrett's esophagus. The line enclosed within the box represents the median value; the small circles indicate outlying data points. EGFR expression in specimens with SIM, I/LGD, HGD and EAC was significantly increased in comparison with that in NB ($P < 0.01$). A significant positive correlation between EGFR expression and histopathologic changes from SIM to EAC was found ($r = 0.97$, $P < 0.001$).

correlation between EGFR expression and histopathologic changes from I/LGD to EAC ($r = 0.97$, $P < 0.001$) (Figure 4).

Correlation between COX-2 and EGFR in metaplasia–dysplasia–adenocarcinoma sequence

The data from computer quantification of COX-2 expression and EGFR expression in all immunohistochemically stained sections were used to determine the correlation between COX-2 and EGFR in the metaplasia–dysplasia–adenocarcinoma sequence. A significant positive correlation between COX-2 expression and EGFR expression was determined in the BE tissues ($r = 0.94$, $P < 0.001$) (Figure 5).

DISCUSSION

Our results showed a detailed information on localization of COX-2 and EGFR protein within the metaplastic cells, dysplastic cells and cancer cells. We demonstrated a significant increase in both COX-2 and EGFR expressions in the metaplasia–dysplasia–adenocarcinoma sequence. In particular, weak COX-2 and EGFR stainings were found in the normal squamous epithelium and NB epithelium, while strong COX-2 and EGFR stainings were found in the region with histological changes of metaplasia, dysplasia and EAC. To our best of knowledge, this is the first study to examine the relationship between COX-2 and EGFR in respect to the histologic progression from BE

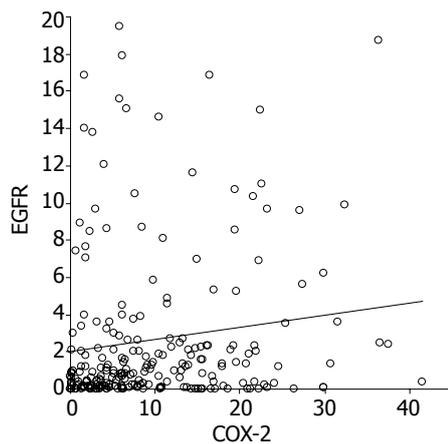


Figure 5 Relationship between COX-2 expression and EGFR expression in 104 patients. A significant positive correlation between COX-2 expression and EGFR expression was found ($r=0.94$, $P<0.001$).

to EAC. Since both COX-2 and EGFR expression levels increase dramatically in BE, each of these changes could enhance the tumorigenic potential of BE^[10,11,18,22].

This study focused on the morphological features of Barrett's epithelium with the expression of COX-2 and EGFR for the following reasons. First, evaluation from BE to EAC is primarily based on the cytomorphological characteristics^[1,7,23,24]. Neoplasia develops in a series of steps through the development of a morphologically changed cell population. It is widely accepted that COX-2 and EGFR play important roles during carcinogenesis, and the over-expression of COX-2 and EGFR would be expected to predispose the epithelium to tumorigenic morphological change. Second, up-regulations of COX-2 and EGFR have been reported in BE and EAC, but no evidence has been provided for the joint signaling of COX-2 and EGFR in the induction of Barrett's epithelium to adenocarcinoma transition. This induction remains an integral process occurring during critical phase of tumor progression. Third, although COX-2 and EGFR play different biological roles in tumor formation, targeting both COX-2 and EGFR to abrogate both pathways and their downstream targets achieved significant antitumor and antiangiogenic effects in the *in vivo* and *in vitro* studies^[21,25]. Therefore, the two proteins might be cooperatively carcinogenic, which intensify the transformation effects on Barrett's metaplasia to dysplasia to adenocarcinoma sequence.

The exact function of COX-2 in tumor development and progression *in vivo* is not known as yet. One possibility follows that over-expression of COX-2 leads to high levels of prostaglandins synthesis in the tissues. Prostaglandins produced by COX-2 may subsequently facilitate tumor progression by acting as growth factors, differentiation factors, immunosuppressors and angiogenic agents^[26,27]. Other possibilities from recent studies indicate that reactive oxygen species (ROS) are generated during the conversion from prostaglandins G2 to prostaglandins H2^[28,29], this enhanced level of ROS parallels the levels of COX-2 mRNA. The COX-2-derived oxidative DNA damage may contribute to the accumulation of genetic damage, and the accumulation of genetic and epigenetic aberrations produces one or more clones with tumorigenic potential.

In addition, the COX-2 enzyme itself may promote tumor development and progression by a prostaglandin-independent pathway. This is supported by the evidences that NSAIDs suppressed proliferative activity and inhibited the growth in colon cancer cells without repressing prostaglandins synthesis^[30]. Over-expression of COX-2 can also up-regulate anti-apoptotic oncoprotein such as BCL-2 and multiple proliferation-promoting cyclins and cyclin-dependent kinases, and down-regulate some tumor suppressor proteins^[17,31]. The postulation for the involvement of COX-2 over-expression related to the progression in metaplasia to dysplasia to adenocarcinoma sequence is based on extensive studies of the relationship between COX-2 and tumor development and progression in multiple organ system.

Our data demonstrated a strong immunoreactivity for COX-2 protein within metaplastic cells, dysplastic, and malignant cells when compared to normal squamous cells and NB columnar cells. The cytomorphological features of COX-2 distribution in all epithelium may reflect two aspects of the effect of COX in contribution to the development of tumor. COX-2 is an important factor to maintain the microenvironment for tumor growth, and COX-2 may not be the critical factor in contribution to the phenotype changes of pre-malignant cells. COX-2 is undetectable in most tissues in the physiological condition^[13,14]; the animals lacking COX-2 and prostaglandins synthesis show no innate gastrointestinal pathology^[32]. These features of COX-2 do not confirm COX-2 expression as a central event in carcinogenesis. On the other hand, it is also possible that duodenal-gastro reflux and/or rapid growth and invasion of cancer cells in BE would stimulate local inflammation to inflammatory mediators and mitogens^[12,13], which, in turn, would induce COX-2 synthesis. Nevertheless, the high level of COX-2 synthesis would prolong the survival of these abnormal cells and increase the chance for tumorigenesis.

EGFR signaling module plays a fundamental role in the morphogenesis of a diverse spectrum of organisms, and has been highly conserved through the course of evolution. In humans, the EGFR family, which consists of the EGFR itself and the receptors known as HER 2-4, and more than 30 ligands lie at the head of a complex, multi-layered signal-transduction network^[33]. The aberrant activity of the members in this family has been shown to play a key role in the development and growth of tumor cells^[9]. The EGFR family is over-expressed in various types of human neoplasms, such as esophagus, thyroid, breast, testis, bladder, melanoma, pancreas, colon, cervical carcinomas, renal carcinoma, prostate and liver cancer^[10]. The underlying mechanism for EGFR involvement in carcinogenesis is likely to reduce the requirement for exogenously supplied growth factors to maintain tumorigenic cells proliferation. Over-expression of EGFR can hypersensitize the cells to low concentration of growth factors to maintain their viability, and to facilitate their phenotype transformation mediated by these growth factors^[34,35]. The aberrant activity of the EGFR might regulate the expression of specific oncogenes or tumor suppressor genes involved in the intracellular signal transduction pathway to synthesize endogenously produced growth factors^[36]. Several stud-

ies have also supported the notion that the endogenous growth factors might function via an intracrine, juxtacrine, autocrine or paracrine mechanisms to control cell proliferation^[34-36].

The results obtained from this study for EGFR staining showed a significant effect of EGFR in contribution to the cytomorphological changes. A large amount of EGFR protein was expressed in the epithelial cells displaying multiple cell layers, enlarged nucleus and less cytoplasm in SIM, I/LGD, HGD and EAC. This over-expression was consistent across the BE progression, thereby indicating an important role of EGFR in the initiation of Barrett's cells transition to adenocarcinoma. The phenotypic effects of abnormal function of EGFR protein are far more dramatic. Recent studies have indicated that animals lacking EGFR have abnormal eyes and epidermal tissues and die due to defects in the development of epithelial organ^[37,38]. Therefore, EGFR may play a key role in the morphogenesis of metaplasia, dysplasia and adenocarcinoma. In our study, the immunoreactivity of EGFR was observed in the same increasing trend with the metaplasia to dysplasia to adenocarcinoma sequence as that seen in COX-2 immunoreactivity, thus suggesting a cooperative effect of these two proteins.

In summary, our study demonstrates a positive relationship between expression of COX-2 and EGFR in the progression from metaplasia to dysplasia to adenocarcinoma in subjects with BE. This suggests that both COX-2 and EGFR may increase the likelihood of Barrett's cells to undergo abnormal cell cycling or gene expression. Further studies are needed to correlate the COX-2 and EGFR expression pattern with clinical outcome. Animal models and tissue culture are also required to determine the exact mechanism of COX-2 and EGFR action in the metaplasia to dysplasia to adenocarcinoma sequence.

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Low central venous pressure reduces blood loss in hepatectomy

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CONCLUSION: LCVP is easily achievable in technique. Maintenance of CVP ≤ 4 mmHg can help reduce blood loss during hepatectomy, shorten the length of hospital stay, and has no detrimental effects on hepatic or renal function.

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Key words: Hepatectomy; Hepatocellular carcinoma; Central venous pressure; Blood loss

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Abstract

AIM: To investigate the effect of low central venous pressure (LCVP) on blood loss during hepatectomy for hepatocellular carcinoma (HCC).

METHODS: By the method of sealed envelope, 50 HCC patients were randomized into LCVP group ($n=25$) and control group ($n=25$). In LCVP group, CVP was maintained at 2-4 mmHg and systolic blood pressure (SBP) above 90 mmHg by manipulation of the patient's posture and administration of drugs during hepatectomy, while in control group hepatectomy was performed routinely without lowering CVP. The patients' preoperative conditions, volume of blood loss during hepatectomy, volume of blood transfusion, length of hospital stay, changes in hepatic and renal functions were compared between the two groups.

RESULTS: There were no significant differences in patients' preoperative conditions, maximal tumor dimension, pattern of hepatectomy, duration of vascular occlusion, operation time, weight of resected liver tissues, incidence of post-operative complications, hepatic and renal functions between the two groups. LCVP group had a markedly lower volume of total intraoperative blood loss and blood loss during hepatectomy than the control group, being 903.9 ± 180.8 mL vs $2\ 329.4 \pm 2\ 538.4$ ($W=495.5, P<0.01$) and 672.4 ± 429.9 mL vs $1\ 662.6 \pm 1\ 932.1$ ($W=543.5, P<0.01$). There were no remarkable differences in the pre-resection and post-resection blood losses between the two groups. The length of hospital stay was significantly shortened in LCVP group as compared with the control group, being 16.3 ± 6.8 d vs 21.5 ± 8.6 d ($W=532.5, P<0.05$).

INTRODUCTION

Hepatectomy remains as the treatment of choice for hepatocellular carcinoma (HCC). Intra-operative blood loss is one of the major causes for post-operative morbidity and mortality. Various techniques, such as Pringle's maneuver and unilateral hepatic hilum occlusion, have been used to control bleeding from hepatic arterial and portal venous systems during hepatectomy in clinical settings. However, for excision of HCC adjacent to major blood vessels, hepatic system could be the major source of hemorrhage, especially after the application of Pringle's maneuver. Hence, effective control of hepatic venous hemorrhage is crucial to minimize intraoperative blood loss. This prospective randomized clinical trial aims at evaluating the role of low central venous pressure (LCVP) in reducing blood loss during hepatectomy for HCC.

MATERIALS AND METHODS

Patients' data and grouping

From June 2002 to December 2003, a total of 50 consecutive patients with HCC (40 men and 10 women) underwent hepatectomy by the same group of surgeons at our hospital. By the sealed envelope method, the patients were blindly randomized into LCVP group ($n=25$) and control group ($n=27$) at the beginning of the operation. Two patients in the control group were excluded from the study because hepatectomy was given up due to cardiac arrest in one and unclear tumor demarcation in the other. Eventually, there were 25 patients in each group. The demographic data of the patients are shown in Table 1,

Table 1 Comparison of patient's clinical data between two groups

| Groups | Gender (male/female) ¹ | Age (yr) ² | Body weight (kg) ³ | Liver cirrhosis (n) ⁴ | Liver function (Child-Pugh class) ⁵ | | |
|---------|-----------------------------------|-----------------------|-------------------------------|----------------------------------|--|---|---|
| | | | | | A | B | C |
| LCVP | 19/6 | 45.33±14.59 | 60.11±12.14 | 14 | 21 | 3 | 1 |
| Control | 21/4 | 46.00±12.14 | 59.91±10.84 | 15 | 21 | 4 | 0 |

¹ $\chi^2=0.500$, ² $t=0.174$, ³ $t=0.055$, ⁴ $\chi^2=0.082$, ⁵ $\chi^2=1.143$.

Table 2 Comparison of blood routine examination, hepatic and renal functions, and coagulation function between two groups

| Groups | Blood routine examination | | | Hepatic function | | | Coagulation function | Renal function | |
|----------|---------------------------|---------------------------|------------|------------------|--------------------|------------|----------------------|---------------------------|------------------|
| | Hb(g/L) | RBC($\times 10^{12}$ /L) | HCT | ALT(μ /L) | TBIL(μ mol/L) | ALB(g/L) | PT(s) | BUN ^a (mmol/L) | Cr(μ mol/L) |
| LCVP | 134.08±19.17 | 4.632±0.83 | 0.41±0.058 | 51.24±29.91 | 29.04±42.71 | 41.29±6.13 | 12.56±1.12 | 4.75±1.24 | 69.04±15.72 |
| Control | 136.48±13.51 | 4.58±0.55 | 0.41±0.039 | 61.36±47.51 | 22.64±11.27 | 41.12±4.79 | 12.53±1.16 | 5.48±1.12 | 70.76±17.99 |
| <i>t</i> | 0.512 | 0.292 | 0.233 | 0.901 | 0.724 | 573.00 | 0.087 | 2.096 | 0.360 |

^a $P<0.05$, BUN values in both groups were within normal range (2.5-6.4 mmol/L).

and the preoperative laboratory findings are demonstrated and listed in Table 2.

Intraoperative management

In the LCVP group, patient's right internal jugular vein was cannulated by a dual-channel catheter following anesthesia, with one channel being connected to the transducer for continuously monitoring CVPs. Arterial pressure was measured by puncture of radial artery. Systolic blood pressure (SBP) was maintained above 90 mmHg by intravenous infusion of dopamine (4-6 μ g/kg). During hepatectomy, CVP was maintained at 2-4 mmHg by adopting Trendelenburg's posture (with head 15° lower than low extremities), limiting the volume of infusion and intravenously administering nitroglycerine (0.5-2 mg/time), and furosemide (10-20 mg) was used if necessary. From the beginning of anesthesia to the completion of liver resection, the infusion was maintained at a speed of < 75 mL/h, urine output > 25 mL/h, and SBP > 90 mmHg. If hypotension occurred, the dose of dopamine and the speed and volume of infusion were adjusted. If hemoglobin (Hb) was < 80 g/L during the operation, packed red cells were transfused. After liver resection and hemostasis were completed, the blood volume was restored with crystalloid and colloid solutions. Post-operative Hb value should be kept at above 100 g/L. In the control group, patients were managed in the same way as those in LCVP group, except for manipulation of patient's posture and administration of nitroglycerine and furosemide to lower CVP.

Hepatectomic technique

The liver was completely separated. The extent of hepatectomy and whether or not applying hepatic blood inflow occlusion were determined based on the tumor situation and severity of liver cirrhosis. For the patients with severe cirrhosis or peripheral tumors, hepatic

blood inflow occlusion was not used. For patients with tumor located at one lobe of the liver, unilateral hepatic hilum occlusion was employed. In hepatic parenchymal transection, method of blunt division but not cavitron ultrasonic surgical aspirator (CUSA) was used. The ductal structures were first ligated and then divided, with reinforced suture for larger blood vessels. The cutting surface of the liver was sprayed with biological glue. The patterns of hepatectomy between the two groups were compared as shown in Table 3.

Measurement of blood loss during hepatectomy

Total intra-operative blood loss consisted of the following three parts: (1) from the start of the operation to complete separation of the liver (pre-hepatectomic blood loss); (2) during division of hepatic parenchyma (intra-hepatectomic blood loss); (3) from completion of hepatic parenchymal division to the closure of the abdominal wall (post-hepatectomic blood loss). Operation was continued to the next step only after complete hemostasis was achieved. The blood-collection bottle was changed in each step and the volume of the collected blood was measured. All blood-stained gauzes were harvested and weighed. The difference between the weight of the blood-stained gauzes and their initial weight was considered as the weight of blood in the gauze, which was then converted into the volume of blood (the blood specific gravity: 1.054-1.062 g/mL in men with an average of 1.058 g/mL; 1.048-1.062 g/mL in women with an average of 1.055 g/mL).

Evaluation parameters

The following pre-, intra-, and post-operative data were collected: (1) patient's gender, age, body weight, red blood cell (RBC) count, hematocrit (HCT), hemoglobin (Hb), alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL), serum albumin (ALB), blood urea nitrogen (BUN), creatinine (Cr), prothrombin

Table 3 Comparison of operative pattern between two groups

| Groups | Types of hepatectomy ¹ | | | | Types of vascular occlusions (n) ² | | Maximal tumor dimension (cm) ⁴ | Weight of the resected liver tissues (g) | |
|---------|-----------------------------------|------|---------------------|-------------------------|---|------------------|---|--|---------------|
| | Right | Left | ≥2 hepatic segments | Single hepatic segments | Unilateral | Portal hepatitis | | | |
| LCVP | 5 | 3 | 13 | 4 | 4 | 7 | 20.00±12.17 | 10.7±4.9 | 633.80±567.38 |
| Control | 4 | 5 | 10 | 6 | 2 | 11 | 17.27±6.56 | 9.88±4.2 | 603.20±497.62 |

¹ $\chi^2=1.402$, ² $\chi^2=1.709$, ³ $t=0.621$, ⁴ $t=0.633$, ⁵ $t=0.203$.

Table 4 Comparison of intra-operative blood loss between two groups

| Group | Pre-hepatectomy | Intra-hepatectomy | Post-hepatectomy ² | Total |
|----------------|-----------------|--------------------------|-------------------------------|--------------------------|
| LCVP | 318.2±605.6 | 672.4±429.9 ^b | 42±35.4 | 903.9±180.8 ^d |
| Control | 603.4±973.7 | 1 662.6±1 932.1 | 63.4±52.9 | 2 329.4±2 538.4 |
| W ¹ | 495 | 543.5 | 538 | 495.5 |

¹Rank sum test; ² $P=0.05$, ^b $P<0.01$, ^d $P<0.01$ vs control.

time (PT), presence or absence of liver cirrhosis and liver function grading; (2) pre-hepatectomic, intra-hepatectomic and post-hepatectomic blood loss and total intra-operative blood loss; (3) operation time, pattern of hepatic hilum occlusion and duration, maximal tumor dimension, weight of the excised liver tissue and extent of hepatectomy, transfusion volume of packed RBCs and fresh frozen plasma (FFP); (4) liver and renal functions on post-operative d 1, 3, and 7; (5) post-operative morbidity and length of hospital stay.

Statistical analysis

The results were expressed as mean ± SD. SPSS software package version 10.0 was used to run the statistical analysis. For continuous variables, Student's *t* test, rank test or ANOVA was employed to compare the inter-group difference, and χ^2 test was adopted for categorical variables. Statistical significance was considered at two-tailed $P < 0.05$.

RESULTS

Patient's general clinical data

There were no significant differences in gender, age, body weight, severity of liver cirrhosis and grades of liver function between LCVP and control groups (Table 1).

Preoperative laboratory data

Table 2 shows that preoperative RBC count, HCT, Hb, ALT, AST, TBIL, ALB, PT and Cr in LCVP group was not markedly different from those in the control group. Preoperative BUN in both the groups was within the normal range, being higher in control group than in LCVP group ($P < 0.05$).

Operative pattern

All patients had large tumors. There were no remarkable differences in patterns of operation, modes of hepatic

blood inflow occlusion and duration, maximal tumor dimension, weight of the excised hepatic tissue between the two groups (Table 3).

Operative time, intra-hepatectomic blood loss, blood transfusion, post-operative complications and length of hospital stay

Total intra-operative blood loss and intra-hepatectomy blood loss were dramatically less in LCVP group than in the control group ($P < 0.01$, Table 4). There were no significant differences in pre- and post-hepatectomic blood losses between the two groups (Table 4). Eight of twenty-five patients in LCVP group and 14 of 25 patients in control group needed blood transfusion, with a significantly lower volume of transfusion in LCVP than in the control group ($P < 0.05$, Table 5). There was no significant difference in operative time between the two groups (Table 5). Post-operative complications included biliary fistula, gastrointestinal bleeding, pleural effusion and subphrenic fluid collection, with an incidence of 20% (5/25) in LCVP group and 24% (6/25) in control group ($P > 0.05$). LCVP group had a remarkably shorter hospital stay than the control group, being 16.3±6.8 d vs 21.5±8.6 d ($P < 0.05$).

Post-operative hepatic and renal functions

There were no significant differences in ALT, TBIL, and Cr on post-operative d 1, 3, and 7 between the two groups (Table 6, $P > 0.05$). BUN was significantly higher in control group than in the LCVP group, but it was within the normal range in both groups (Table 6, $P > 0.05$).

DISCUSSION

Blood loss during hepatectomy has an adverse effect on the post-operative outcome and increases post-operative morbidity. Intra- or post-operative massive blood transfusion carries risks of causing infectious diseases,

Table 5 Comparison of operation time and incidence of post-operative complications

| Groups | Operation time (min) ¹ | Intra- and post-operative transfusion (mL) | | Incidence of post-operative complications (n) ⁴ |
|---------|-----------------------------------|--|----------------------------|--|
| | | Packed RBC ² | FFP ³ | |
| LCVP | 229.60±67.33 | 525.00±237.57 ^a | 437.50±250.36 ^c | 5 |
| Control | 246.00±112.36 | 1 285.71±1 162.13 | 1 057.14±658.33 | 6 |

¹t=0.626; ²W=63.00, ³t=2.537, ⁴χ²=9.778, ^aP<0.05, ^cP<0.05 vs control.

Table 6 Comparison of post-operative hepatic and renal functions between two groups

| Parameters | Postoperative day | LCVP | Control |
|----------------------------|-------------------|---------------|---------------|
| ALT (μ/L) ¹ | 1 | 561.00±409.23 | 700.76±610.77 |
| | 3 | 286.4±204.09 | 396.60±423.86 |
| | 7 | 104.60±56.18 | 173.04±268.17 |
| TBIL (μmol/L) ² | 1 | 29.04±42.71 | 22.64±11.27 |
| | 3 | 39.34±47.08 | 40.46±28.33 |
| | 7 | 23.69±26.40 | 28.95±30.82 |
| BUN (mmol/L) ³ | 1 | 4.75±1.24 | 5.48±1.12 |
| | 3 | 4.87±2.59 | 5.72±1.84 |
| | 7 | 5.10±1.75 | 6.03±2.23 |
| Cr (μmol/L) ⁴ | 1 | 69.04±15.72 | 70.76±17.99 |
| | 3 | 58.04±15.68 | 65.28±17.67 |
| | 7 | 59.12±11.57 | 60.96±15.60 |

¹F=72.955, ²F=0.185, ³F=0.860, ⁴F=6.462.

coagulatory disorder, acute respiratory distress syndrome, multiple organ failure, and also promotes tumor recurrence due to its inhibitory effects on immunologic function^[1,2]. Hence, various methods, including Pringle's maneuver, unilateral hepatic hilum occlusion and normothermic total hepatic vascular exclusion^[3], have been adopted to reduce intra-operative blood loss. In recent years, application of CUSA in liver resection under hepatic hilum occlusion has been reported to significantly lessen the intra-operative blood loss^[4]. Some studies reported that LCVP during liver resection could significantly cut down the intra-operative blood loss, decrease the incidence of post-operative complications and shorten the hospital stay^[5,6]. Under hepatic hilum occlusion, blood loss during liver resection mainly derives from hepatic vein and hepatic short vein. Hepatic sinusoidal pressure is directly related to CVP. With lowering of the pressure in inferior vena cava, the hepatic venous pressure and then hepatic sinusoidal pressure would decline. Blood loss during liver resection is proportional to the pressure gradient of vascular walls and the diameter of the injured vessels. Though total hepatic vascular exclusion or clamping inferior vena cava can reduce blood loss from hepatic venous system during hepatectomy^[7], they are associated with remarkable injury and technically difficult.

LCVP in hepatectomy was generally referred to as CVP lower than 5 mmHg^[6]. Maintenance of LCVP should not lower the arterial pressure. In the present study, by maintaining CVP below 5 mmHg during liver resection with manipulation of patient's posture, administration of drugs and control of infusion speed, the arterial pressure

was effectively sustained and blood loss was markedly cut down. Our results suggested that Trendelenburg's posture was helpful to prevent LCVP-induced air embolism. With the maintenance of the arterial pressure, the perfusion of organs was ensured. The post-operative evaluation showed that LCVP would not deteriorate hepatic and renal functions. LCVP group had shorter hospital stay than the control group. It is suggested that LCVP was helpful for post-operative recovery. The LCVP technique was not complicated and achievable even in middle-class hospitals. Hence, it is of clinical value.

Many studies reported that various methods reduced intra-operative transfusion volume^[8]. However, transfusion volume sometimes could not correctly reflect intra-operative blood loss, since the former was related to the patient's preoperative conditions, tolerance to ischemia, control of intraoperative infusion volume and speed, and anesthetist's mastery of indication for transfusion. Intra-operative blood loss included bleeding not only from hepatic parenchymal division, but also from dissection of perihepatic ligaments, separation of tumors from adjacent tissues, the cutting surface of liver after resection and the accidental tumor rupture. The latter was also affected by the surgeon's experiences and techniques, and patient's coagulation function. Hence, strictly speaking, only bleeding from hepatic parenchymal division accounts for the real blood loss of hepatectomy. In the present study, we have divided the procedure of hepatectomy into three steps, i.e.; pre-, intra-, and post-hepatectomic, and blood loss in each step was completely collected and accurately measured. Our results showed that total intra-operative blood loss was markedly less in LCVP group than that in the control group. There were no significant differences in pre-hepatectomic and post-hepatectomic blood losses between the two groups, but intra-hepatectomic blood loss was remarkably lower in LCVP group than in the control group.

It suggested that lowering CVP during hepatectomy could effectively reduce blood loss. Additionally, transfusion volume was markedly cut down in LCVP as compared with the control group. It indicated that decline in intra-hepatectomic blood loss could reduce the transfusion volume, which is helpful for the recovery of patient's immunological function and prevention of tumor recurrence. The present study is a prospective randomized clinical trial. The number of cases is not big enough, and the effect of decline in intra-operative blood loss on prognosis still awaits further observations.

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RAPID COMMUNICATION

Removal selectivity of Prometheus: A new extracorporeal liver support device

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Abstract

AIM: To evaluate whether treatment with the Prometheus[®] system significantly affects cytokines, coagulation factors and other plasma proteins.

METHODS: We studied nine patients with acute-on-chronic liver failure and accompanying renal failure. Prometheus[®] therapy was performed on 2 consecutive days for up to 6 h in all patients. Several biochemical parameters and blood counts were assessed at regular time points during Prometheus[®] treatment.

RESULTS: We observed a significant decrease of both protein-bound (e.g. bile acids) and water-soluble (e.g. ammonia) substances after Prometheus[®] therapy. Even though leukocytes increased during treatment ($P < 0.01$), we found no significant changes of C-reactive protein, interleukin-6, and tumor necrosis factor- α plasma levels (all $P > 0.5$). Further, antithrombin 3, factor II and factor V plasma levels did not decrease during Prometheus[®] therapy (all $P > 0.5$), and the INR remained unchanged ($P = 0.4$). Plasma levels of total protein, albumin, and fibrinogen were also not altered during Prometheus[®] treatment (all $P > 0.5$). Finally, platelet count did not change significantly during therapy ($P = 0.6$).

CONCLUSION: Despite significant removal of protein-bound and water-soluble substances, Prometheus[®] therapy did not affect the level of cytokines, coagulation factors or other plasma proteins. Thus, the filters and adsorbers used in the system are highly effective and specific for water-soluble substances and toxins bound to the albumin fraction.

INTRODUCTION

Extracorporeal liver support devices have recently gained much interest. Prometheus[®] is a newly designed system for extracorporeal treatment of liver failure. It is based upon a recently developed method of albumin dialysis, i.e. fractionated plasma separation and adsorption^[1]. First, an albumin-permeable membrane (AlbuFlow, cut-off 250 ku) separates the albumin fraction together with all albumin-bound substances in a secondary circuit. There, direct purification of the plasma is performed by one or two adsorbers (Prometh01 and Prometh02) in order to remove albumin-bound toxins. In parallel, a conventional high-flux dialysis is performed to remove the water-soluble substances.

Results from past studies have documented that extracorporeal liver support devices can remove cytokines and other important plasma proteins^[2,3]. Theoretically, removal of these substances may influence spontaneous liver regeneration, similarly as has been proposed for interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α)^[4]. Furthermore, development of thrombopenia with an increased risk of bleeding was documented in several patients undergoing albumin dialysis using the MARS system^[5]. Until now, there are not sufficient controlled studies available that have evaluated whether treatment with the Prometheus[®] device may result in an unspecific removal of cytokines or leads to a decrease of coagulation factors or platelets. In addition, albumin or other plasma proteins may be lost during Prometheus[®] as a result of removal or after clotting in the secondary circuit. Therefore, we performed a prospective clinical study in patients with liver failure and assessed the course of different cytokines, coagulation factors, protein fractions, and thrombocytes during treatment with Prometheus[®].

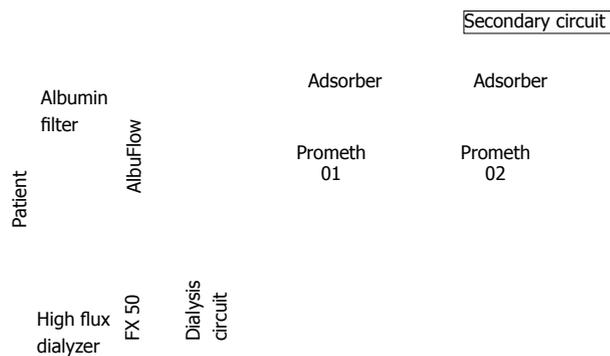


Figure 1 The albumin fraction of the patient's blood is filtered through an albumin-permeable membrane (AlbuFlow[®]) into a secondary circuit. There, the separated albumin fraction is purified by direct adsorption of albumin-bound toxins using one or two adsorbers (Prometh01[®] and Prometh02[®]). The plasma is then pumped back into the primary circuit where a conventional high-flux dialysis (FX 50) is performed.

MATERIALS AND METHODS

Patients

The study was approved by the Ethics Committee of the Hannover Medical School. The Declaration of Helsinki and the rules from Good Clinical Practice were followed. Written informed consent was obtained from all patients or next of kin. Nine patients (four males) with liver failure (five with end-stage cirrhosis, two with acute alcoholic hepatitis, two with liver failure late after liver transplantation) and concomitant renal failure were included in the trial. Their mean age was 52 ± 8 years, and the average APACHE II score was 16.8 ± 3.2 . Seven patients (78%) had the highest stage of cirrhosis (C Child-Pugh score) with an average of 11.3 ± 1.7 points. Six patients (67%) were treated in the intensive care unit. All patients were already undergoing hemodialysis and had a functionable double-lumen catheter in place.

Prometheus[®] device

Blood coming from the patient by a double-lumen dialysis catheter is pumped into the extracorporeal circuit. There, it passes through an albumin-permeable filter, which is made of polysulfone (AlbuFlow[®]). The filter has a sieving coefficient that is specially designed. It allows large molecules up to the size of albumin (68 ku) to pass into the secondary circuit. The cut-off is at about 250 ku. Larger molecules cannot pass through the filter. The secondary circuit is filled with isotonic sodium chloride solution. It is driven by a separate plasma pump. By recirculation of the albumin-rich plasma, the filtration and adsorption capacities are enhanced. One or two adsorbers are used to directly purify the filtered albumin-rich plasma: a neutral resin adsorber (Prometh01[®]) for direct adsorption of the albumin-bound toxins and an anion exchanger (Prometh02[®]) with chloride used as counterion. The content of the adsorber consists of spherical adsorber particles with 300-1 000 μm in diameter. The adsorbers consist of styrene-divinylbenzene copolymer. Their cylindrical housing has a central inlet and outlet closed

at both ends by sieves with a mesh size of 100 μm . The sieves circumvent a passage of the particles through the inlets. Even in case of leakage, they are held back in the secondary circuit by the albumin filter membrane. After the purification of the fractionated plasma in the secondary circuit, the purified plasma is returned to the primary circuit, i.e. into the blood side of the albumin filter. Here the blood is guided through a high-flux hemodialyzer (FX 50[®]). There, water-soluble substances are removed by diffusion.

A modified hemodialysis unit (4008 H) is used to integrate the two parts of the Prometheus[®] system (Fresenius Medical Care AG, Bad Homburg, Germany): a "standard" extracorporeal blood circuit connected to the double-lumen dialysis catheter and a secondary circuit containing the separated plasma (Figure 1). Both circuits are separately monitored by different hardware and software allowing the user to perform hemodialysis with simultaneous albumin detoxification or conventional hemodialysis alone.

Treatments

Instead of their regular hemodialysis sessions, all patients underwent two Prometheus[®] treatments for at least 4 h on two consecutive days. In 5/9 patients (56%), the device was equipped with two adsorbers (Prometh01[®] and Prometh02[®]), while the other four patients (44%) were treated only with Prometh01[®]. The average treatment time was 5.1 ± 1.1 h, and mean blood flow rate was 193 ± 10 mL/min. The total processed volume within each treatment was 58 ± 14 L. Anticoagulation during therapy consisted of unfractionated heparin administered as a bolus. Because of the increased risk of bleeding in liver patients, heparin administration was restricted to low doses. The activated coagulation time was used to control anticoagulation. It was targeted between 120 and 150. Blood samples for measurement of cytokines (TNF- α and IL-6), platelets, coagulation factors (antithrombin 3, factors II and V) and plasma proteins (total protein, albumin, and fibrinogen) were taken before the start of Prometheus therapy, after 30 min, 2, and 5 h of treatment as well as 24 h after the end of the treatment.

Measurements

Plasma concentration of C-reactive protein was measured with a clinically validated nephelometric assay (Dade Behring, Germany); the normal range is below 5 mg/L. Plasma concentrations of inflammatory cytokines, e.g. IL-6 and TNF- α , were measured with an ELISA (R&D Systems, USA). The intra-assay coefficients of variation for these tests ranged between 4.9% and 6.8%, respectively. The other biochemical parameters were measured by certified routine analytical methods.

Statistical analysis

The SPSS program was used for statistical analysis (version 12.0, SPSS, Chicago, USA). All data are presented as mean \pm SD. Pre- and post-treatment values were compared using two-tailed *t*-test for paired data. Correlations were assessed with Pearson's correlation analysis. A $P < 0.05$ was considered as statistically significant.

RESULTS

A significant decrease of both protein-bound substances (e.g., bile acids; $P < 0.01$) and water-soluble substances (e.g., urea; $P < 0.001$) was observed under Prometheus[®] therapy (Figure 2A). Even though leukocytes increased from 15.1×10^3 to $22.7 \times 10^3/\mu\text{L}$ ($P < 0.01$), there were no significant changes of plasma concentrations of C-reactive protein ($P = 0.7$), TNF- α ($P = 1.0$; Figure 2B) or IL-6 ($P = 0.9$; Figure 2B). TNF- α and IL-6 were correlated with each other ($P < 0.03$). Furthermore, they were positively associated with body temperature (TNF- α : $P < 0.03$; IL-6: $P < 0.001$), but not with C-reactive protein (both $P > 0.2$). All these parameters were already increased in our patients before Prometheus[®] therapy.

Platelet count and coagulation factors such as antithrombin 3, factors II and V were substantially decreased in our patients before the start of Prometheus[®] therapy. However, there was no decrease of platelets during treatment ($P = 0.6$). Furthermore, the coagulation factors and INR did not significantly change under Prometheus[®] treatment (all $P > 0.4$; Figure 2C). All coagulation factors were highly associated with each other (all $P < 0.001$) and with C-reactive protein (all $P < 0.01$). A strong positive association was found between factors II and factor V on one hand and leukocyte count on the other hand (both $P < 0.001$).

Plasma levels of total protein, albumin, and fibrinogen were reduced in liver patients before the start of Prometheus[®] therapy. Even though we had two episodes of clotting of the secondary, i.e. albumin-filled circuit occurred, we observed no significant alterations of total protein, albumin, and fibrinogen during treatment (all $P > 0.5$; Figure 2D).

In one patient with hepatitis C cirrhosis and hepatorenal syndrome, we found a marked increase of TNF- α (from 17 to 12 420 ng/L) and IL-6 (from 49 to 15 732 ng/L) already after the 2nd h of treatment on the 1st day of Prometheus[®] therapy. In parallel, leukocytes decreased from 8.1×10^3 to $1.6 \times 10^3/\mu\text{L}$. No specific clinical problems were recorded at that time, so that the Prometheus[®] treatment was completed. When treatment was stopped, mean arterial pressure was 52 mmHg, whereas body temperature was 38.9°C and leukocytes $4.4 \times 10^3/\mu\text{L}$. At this time point, we later measured already decreasing TNF- α plasma levels (1 012 ng/L), whereas IL-6 was still increasing (76 250 ng/L). This episode turned out to be a catheter-related bacteremia, and the catheter was removed immediately. After the catheter had been changed, no clinical problems were observed during Prometheus[®] therapy on the next day. Plasma levels of TNF- α after the second treatment decreased to 49 ng/L and that of IL-6 to 108 ng/L.

DISCUSSION

Removal selectivity of extracorporeal blood purification devices is a key to assess their specificity and efficacy. Therefore, it is crucial to evaluate whether particular substances are removed or not. With Prometheus[®] both protein-bound and water-soluble substances that accumulate in liver failure are removed in a single step, i.e. during

a single passage of the blood through the extracorporeal circuit. The system is based upon the FSPA method, and in contrast to the MARS system, Prometheus[®] allows direct purification of the albumin fraction in the secondary circuit^[1]. Furthermore, a high-flux dialyzer is incorporated in the Prometheus[®] system as well.

We were able to show that Prometheus[®] significantly removed protein-bound (e.g., bile acids) and water-soluble substances (e.g., urea), but we did not observe (unspecific) removal of cytokines such as TNF- α and IL-6. In contrast, it has been shown that both cytokines are removed during MARS treatment^[2,3]. This could be of considerable clinical importance, since these pro-inflammatory cytokines are postulated to play a major role also in the process of liver regeneration^[4]. Thus, while cytokine removal may have beneficial effects in clinical conditions where activation of pro-inflammatory cytokines is part of the pathophysiological basis of the syndrome, e.g. systemic inflammatory response syndrome (SIRS)^[7], it could be potentially even harmful in liver patients by impeding liver regeneration^[8]. Since large prospective controlled trials on survival are lacking, it remains to be elucidated whether (unspecific) cytokine removal in liver patients is beneficial or whether these patients benefit more if pro-regenerative cytokines such as TNF- α and IL-6 remain in the body.

Another point of interest in our study was the influence of Prometheus[®] therapy on coagulation factors and platelets. Usually, patients with liver failure present with moderate to severe coagulopathy. In order to prevent bleeding complications in these patients, the coagulation status should not be further worsened by a removal, activation or consumption of coagulation factors. In none of our patients we observed a decrease of platelets, and coagulation factors such as antithrombin 3, factor II or factor V remained stable throughout the therapy without substitution. This is in contrast to data obtained with the MARS system; it may induce thrombopenia in a significant number of patients with a subsequently elevated risk of bleeding complications^[5].

An important point in the evaluation of extracorporeal liver support systems is the possible loss of plasma proteins during treatment. This may result from an unspecific removal, from adsorption on the adsorber, or by a clotting of the secondary circuit that is filled with the patients' albumin. Even though two episodes of clotting of the secondary circuit occurred in our study, no significant loss of plasma total protein or albumin developed. Furthermore, the selectivity of protein removal was confirmed by measurement of serum fibrinogen concentrations. Fibrinogen molecules are much larger than albumin and should be held in patients' blood if the sieving coefficient of the membrane used for plasma separation remains unaltered. As expected, fibrinogen levels in our patients remained stable throughout the whole study period.

In conclusion, our data document that the extracorporeal liver support device Prometheus[®] provides selective removal of protein-bound and water-soluble substances, whereas measured cytokines, coagulation factors or plasma protein fractions as well as platelets remain unaltered. In this aspect the system seems to offer an advantage over the MARS system.

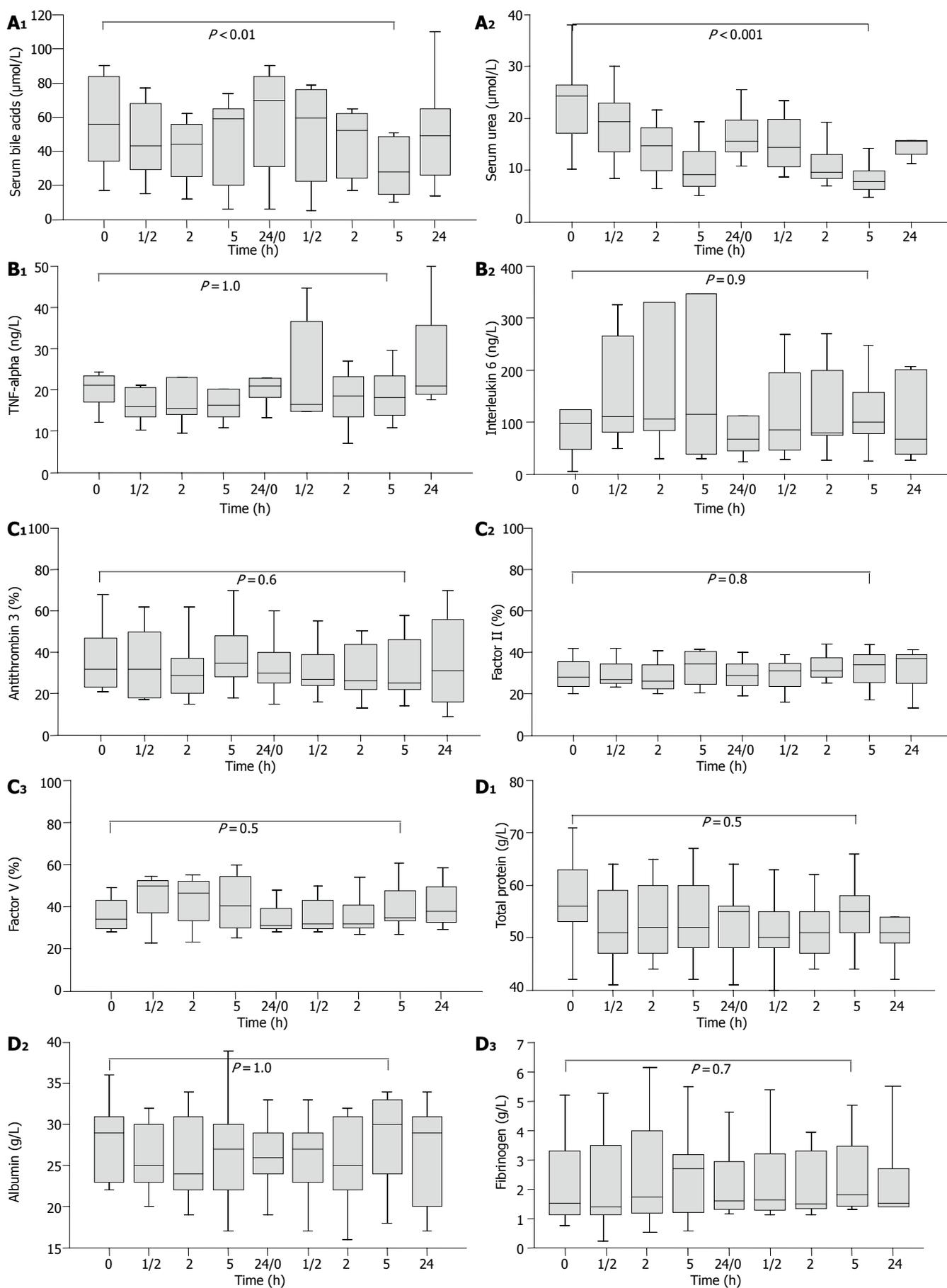


Figure 2 (A1-A2) Course of plasma bile acids and urea concentrations before, during and after 2 d of Prometheus® therapy. Data are presented as median, quartiles, and minimum/maximum values. **(B1-B2)** Course of plasma TNF- α and IL-6 concentrations before, during and after 2 d of Prometheus® therapy. Data are presented as median, quartiles, and minimum/maximum values. **(C1-C3)** Course of antithrombin 3, factors II and V levels before, during and after 2 d of Prometheus® therapy. Data are presented as median, quartiles, and minimum/maximum values. **(D1-D3)** Course of plasma total protein, albumin, and fibrinogen before, during, and after 2 d of Prometheus® therapy. Data are presented as median, quartiles, and minimum/maximum values.

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Randomized controlled trial of consensus interferon with or without zinc for chronic hepatitis C patients with genotype 2

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Abstract

AIM: The beneficial effect of zinc supplementation on the efficacy of interferon as a treatment for chronic hepatitis C had been demonstrated in hepatitis virus genotype 1b of high viral load. This study focused on patients with genotype 2, which is more sensitive to interferon than genotype 1b, and used consensus interferon (CIFN) with or without zinc.

METHODS: We randomized 83 patients with chronic hepatitis C to CIFN at 18 MIU six times/wk for 4 wk, followed by CIFN at 18 MIU six times/wk for another 20 wk, in combination with polaprezinc 300 mg (regimen A, $n=41$) or as monotherapy (regimen B, $n=42$). Thirty-one patients in regimen A and 33 patients in regimen B completed the clinical trial; the remaining patients withdrew because of side effects or a transfer to another hospital.

RESULTS: Sustained biochemical response, defined as a normal aminotransferase level at the end of the 6-mo post-treatment observation, was 68% and 69%, and sustained virological response, defined as undetectable HCV-RNA at the end of the 6-mo post-treatment observation, was 54% and 67% for regimens A and B, respectively.

CONCLUSION: CIFN treatment combined with zinc did not enhance the effect of CIFN as shown by biochemical, virological criteria. No side effects related to polaprezinc were noted.

INTRODUCTION

Interferon (IFN) is an effective treatment for chronic hepatitis C and is the only method of treatment that eliminates hepatitis C virus (HCV)^[1,2]. The incidence of hepatocellular carcinoma (HCC) decreases if liver function improves, even if the virus is not eradicated after IFN treatment^[3-6], or if HCV RNA becomes undetectable during IFN treatment^[7]. IFN treatment for chronic hepatitis C plays a pivotal role in the prevention of HCC.

HCV-RNA clearance rates are reported to be approximately 30-40% in patients treated with IFN alone^[8-10]. However, better results have been reported when IFN- α is used in combination with ribavirin in both naïve patients^[11,12] and in those who failed to respond to, or relapsed after, IFN- α monotherapy^[13,14]. However, IFN plus ribavirin treatment for 24 wk causes more frequent side effects leading to dose reduction or discontinuation of treatment in 28% of cases compared that in 21% of cases in IFN alone treatment^[11]. Furthermore, in Japan, the use of ribavirin is restricted to naïve patients with chronic hepatitis C who have high viral loads greater than 100 KIU/mL.

Consensus IFN (CIFN) is a genetically engineered novel type I IFN molecule derived from commonly observed amino acids of several natural IFN subtypes^[15]. CIFN has demonstrated higher *in vitro* antiviral effects on an equal mass basis, than IFN- 2α or 2β ^[16,17]. In Japan, 9-18 MIU CIFN treatment for 6 mo is reported to be well tolerated and 18 MIU CIFN is considered to be the maximum tolerable dose in patients with chronic hepatitis C and is superior in efficacy, without additional toxicity, to 9 MIU CIFN in high-titer chronic hepatitis C patients^[18].

Serum zinc levels are low in patients with chronic liver disease because of a trace element metabolism disorder^[19]. Previously, we have demonstrated that zinc supplementa-

tion in chronic hepatitis C patients with genotype 1b of high viral load enhanced the response to IFN- α monotherapy^[20]. Although zinc has diverse biological properties including antiviral, anti-inflammatory, and antioxidant functions^[21-24], the mechanism responsible for the beneficial effect of zinc during treatment with IFN remains to be elucidated. Treatment efficacy depends on the genotype, HCV load, and staging; HCV genotypes 2a and 2b are more sensitive to IFN than genotype 1a or 1b^[1,25,26].

We planned a randomized controlled study for the treatment of genotypes 2a and 2b hepatitis C to determine whether zinc supplementation could also enhance the efficacy of IFN in chronic hepatitis C patients as well as genotype 1b. We used CIFN instead of IFN plus ribavirin for the following reasons. (1) Naïve chronic hepatitis C patients with low viral load could not be treated by the standard treatment, IFN- α 2b plus ribavirin or pegylated-IFN plus ribavirin, under the current health insurance regulations in Japan, due to high sensitivity of HCV with low viral load to IFN. (2) Even with high viral loads, HCV genotype 2a/2b can be expected to be relatively sensitive to IFN monotherapy without ribavirin. (3) CIFN has fewer restrictions or contraindications, and is less expensive than the combination of IFN- α 2b and ribavirin.

MATERIALS AND METHODS

Patient selection criteria

Patients with chronic HCV infection who had never received IFN therapy were eligible for this study. The patients were continuously positive for HCV-RNA genotype 2 using the amplicor HCV monitor assay and were diagnosed with chronic active hepatitis from a liver biopsy. The eligibility criteria included histological evidence of chronic hepatitis, as judged on a liver biopsy performed no longer than 6 mo prior to enrolment, and confirmation of HCV infection by polymerase chain reaction (PCR) analysis. Liver-biopsy specimens were assessed independently by two pathologists who were blinded to the patients' clinical details. Pathological diagnosis was made using the classification established by Desmet *et al*^[27]. The exclusion criteria included: age lower than 18 years or older than 65 years, pregnancy or lack of appropriate contraceptive measures in women of child bearing age, previous treatment with antiviral or immunosuppressive drugs, current or previous drug addiction, alcoholism, positive HbsAg or HIV test, histological evidence of liver cirrhosis, concomitant metabolic, autoimmune or neoplastic liver diseases, severe concomitant diseases other than liver disease, history of depression or psychiatric diseases.

Study design

This was a randomized, open-label, controlled study conducted between December 2001 and December 2003. The study was approved by the Ethical Committee of Gunma University. Eighty-three patients gave written informed consent and were randomly assigned to two groups: patients in regimen A received CIFN with polaprezinc and patients in regimen B received CIFN alone. All patients were given CIFN subcutaneously every day for 4 wk and then thrice a wk for 20 wk at a dose of

18 mega units (MU). Forty-one patients in regimen A were given 75 mg of oral polaprezinc twice daily (Plomac; Zeria Pharmaceutical Co., Tokyo, Japan) with CIFN for 24 wk, and zinc administration was discontinued simultaneously with discontinuation of CIFN, for a total of 24 wk. Forty-three patients in regimen B were given CIFN according to the protocol for 24 wk. Serum HCV-RNA was measured before treatment and every mo for one year during and after CIFN therapy. Patient adherence to the protocol and compliance with treatment were encouraged by regular hospital visits.

Endpoints and definitions

Two endpoints were selected for comparing the efficacy of regimen A with the standard regimen B. The first endpoint involved serum HCV-RNA levels. 'Sustained virological response (SVR)' was defined as undetectable HCV-RNA at the end of the 6-mo post-treatment observation. 'End-of treatment virological response (EVR)' was defined as undetectable HCV-RNA at the end of the treatment. 'Non virological response (NVR)' was defined as detectable HCV-RNA at the end of the 6-mo post-treatment observation. The second endpoint was serum alanine aminotransferase (ALT) levels. Normalization of serum ALT at the end of treatment and at the end of the 6-mo post-treatment observation was defined as 'end-of treatment biochemical response (EBR)' and 'sustained biochemical response (SBR)', respectively. 'Non biochemical response (NBR)' was defined as elevation of serum ALT at the end of the 6-mo post-treatment observation.

Assessment of safety

We evaluated all adverse events, subjective and objective symptoms, and laboratory test results for safety. All adverse events were checked by the patients using questionnaires, double-checked, and recorded by the investigators. All adverse events were graded for severity based on the World Health Organization (WHO) criteria and the guideline to grading severity of adverse events determined by the Japanese Ministry of Health and Welfare. Global safety assessments were made at the end of post-treatment observation using the following classifications: no abnormality, abnormality requiring no symptomatic treatments, abnormality requiring symptomatic treatments, abnormality requiring study withdrawal, and non-evaluative.

Statistical analysis

Results were expressed as mean \pm SD of the mean on an intention to treat or per-protocol basis. Statistically significant differences in outcomes between the two regimens were assessed using both parametric and non-parametric tests including the Student's *t*-test for paired and unpaired observations, and Fisher's exact test as appropriate.

A logistic multiple regression model was used to examine the relationship between baseline clinical characteristics and the binary outcome of response to CIFN therapy. Each variable was transformed into categorical data consisting of two simple ordinal numbers for the logistic multiple regression model. Variable selection was an important step in characterizing prognostic relations and identifying variables most strongly related to

Table 1 Characteristics of participating patients

| | Regimen A IFN + zinc | Regimen B (CIFN) | P |
|---|-------------------------|---------------------|----|
| Number of entry | 41 | 42 | |
| Male/Female | 28/13 | 21/21 | NS |
| Age (yr) | 60±8.5 | 55±21 | NS |
| Body mass index | 23±2.4 | 24±3.7 | NS |
| Pre-treatment zinc concentration (µg/dl) | 77.8±13.6 | 82.5±16.4 | NS |
| Pre-treatment ALT (IU/l) | 113±75 | 101±96 | NS |
| Pre-treatment leukocyte counts (×10 ³ mm ⁻³) | 5.1±1.3 | 5.0±1.4 | NS |
| Pre-treatment platelet counts (×10 ³ mm ⁻³) | 19±5 | 17±5 | NS |
| Pre-treatment HCV-RNA genotype (2A/2B) | 31/10 | 31/11 | NS |
| Pre-treatment HCV-RNA titer (KIU/mL) | 484±1075 | 304±318 | NS |
| 0<pretreatment HCV-RNA<100 | 11 | 16 | NS |
| 100≤pretreatment HCV-RNA<400 | 15 | 14 | NS |
| 400≤pretreatment HCV-RNA<700 | 6 | 2 | NS |
| 700≤pretreatment HCV-RNA | 9 | 9 | NS |
| Histological findings (staging) | 1.8±1 | 1.3±0.8 | NS |
| Histological findings (grading) | 1.6±0.7 | 1.3±0.9 | NS |

Mean±SD; NS: not significant

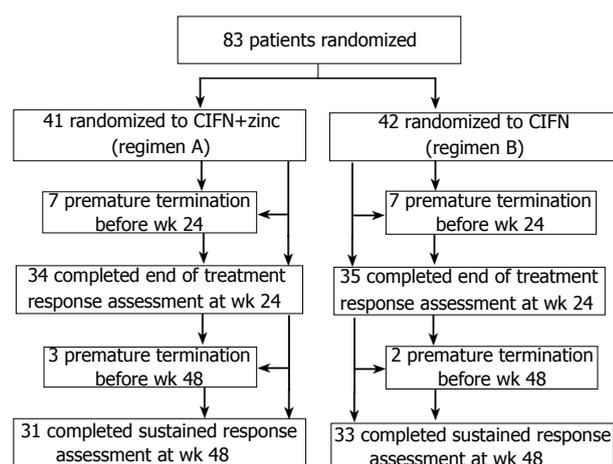


Figure 1 Trial profile.

the outcome. We selected, as candidate predictors of SVR, age, sex, weight, body mass index (BMI), pretreatment platelet counts, pretreatment ALT, pretreatment γ GTP levels, liver histology (staging and grading), pretreatment HCV-RNA viral load, wk of HCV-RNA clearance (less than 1, 4, 8, and 12 wk) and zinc supplementation. Thus, variables ($P < 0.2$) on univariate analysis, that is, zinc supplementation, BMI, staging, pretreatment HCV-RNA viral load (less than 400 KIU/mL), wk of HCV-RNA clearance (less than 8 wk) and pretreatment ALT were included in a logistic multiple regression model. Differences with a $P < 0.05$ were considered significant.

RESULTS

Eighty-three patients were enrolled into the trial; 41 were randomized to regimen A and 42 to regimen B. The pretreatment clinical characteristics of the patients assigned to the two regimens were similar (Table 1).

Table 2 Effect on viremia and serum transaminases

| | Regimen A (CIFN + zinc) | Regimen B (CIFN) | P |
|---|----------------------------|---------------------|----|
| ALT (U/l): | | | |
| Wk 0 | 113±75 | 101±96 | NS |
| Wk 24 | 46±51 | 40±30 | NS |
| Wk 48 | 26±17 | 22±12 | NS |
| Biochemical response (by intention to treat): | | | |
| Wk 24 (EBR) | 46% | 50% | NS |
| Wk 48 (SBR) | 68% | 69% | NS |
| Virological response (by intention to treat): | | | |
| Wk 24 (EVR) | 68% | 76% | NS |
| Wk 48 (SVR) | 54% | 67% | NS |

Mean±SD; EBR: End-of treatment biochemical response; SBR: Sustained biochemical response; EVR: End-of treatment virological response; SVR: Sustained virological response; NS: not significant

Follow-up and protocol compliance

The progression of randomized patients through the trial is summarized in Figure 1. Seven patients in regimen A withdrew prematurely from the study before wk 24 due to intolerance to IFN in five patients, and personal problems or low compliance in the other two patients. Seven patients in regimen B also withdrew prematurely from the study before wk 24 due to intolerance to IFN in six patients, and low compliance in the other patient. Three more patients, all in regimen A, withdrew prematurely from the study before wk 48 because of job transfer or personal problems. Two patients in regimen B withdrew prematurely due to personal problems.

Effect of treatment on viremia and serum transaminase

There were 28/41 (68%) end-of treatment virological responders for regimen A and 32/42 (76%) for regimen B when analyzed on an intention to treat basis; the corresponding values were 28/31 (90%) and 32/33 (97%), respectively, when analyzed on a per-protocol basis (Table 2). There were 22/41 (54%) sustained virological responders for regimen A and 28/42 (67%) responders for regimen B when analyzed on an intention to treat basis; the corresponding values were 22/31 (71%) and 28/33 (85%), respectively, when analyzed on a per-protocol basis. There were 19/41 (46%) end-of treatment biochemical responders for regimen A and 21/42 (50%) responders for regimen B when analyzed on an intention to treat basis; the corresponding values were 19/31 (61%) and 21/33 (64%), respectively, when analyzed on a per-protocol basis. There were 28/41 (68%) sustained biochemical responders for regimen A and 29/42 (69%) responders for regimen B when analyzed on an intention to treat basis; the corresponding values were 28/31 (90%) and 29/33 (88%), respectively, when analyzed on a per-protocol basis. There were no significant differences between the two regimens in these four responses.

Factors contributing to sustained virological response

Variables that correlated with sustained virological response were analyzed by both univariate and multivariate analyses. In univariate analysis, the sustained virological response

Table 3 Logistic regression model of predictors of response to CIFN treatment

| Variables | Odds ratio (95% Confidence Interval) | |
|--|--------------------------------------|--------|
| Zinc supplementation (regimen B vs. A) | 0.254 (0.033-1.948) | 0.1873 |
| BMI (<23.4 vs. ≥23.4) | 1.245 (0.162-9.579) | 0.8330 |
| Staging (0-1 vs. 2-3) | 0.213 (0.024-1.877) | 0.1635 |
| pretreatment HCV-RNA (<400 vs. ≥400) | 0.104 (0.011-0.951) | 0.0450 |
| Week of HCV-RNA clearance (<8 vs. ≥8) | 0.106(0.005-2.399) | 0.1584 |
| pretreatment ALT (<107 vs. ≥107) | 13.709 (0.960-195.749) | 0.1873 |

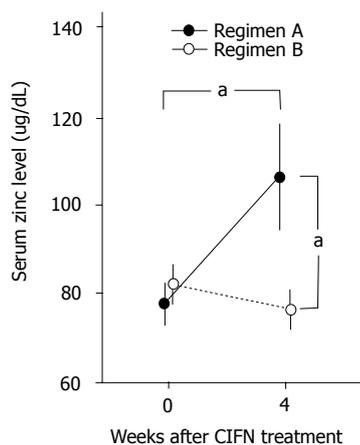


Figure 2 Serum zinc concentrations (mean±SD) at wk 0 (baseline) and after 4 wk of CIFN treatment in patients with regimen A (closed circles) and regimen B (open circles). Serum zinc levels at wk 4 were significantly higher in regimen A patients than those in regimen B patients (^a*P*<0.05). Serum zinc levels at wk 4 were significantly higher than those recorded at wk 0 (baseline) in regimen A patients (^a*P*<0.05).

correlated significantly with liver histology (staging) and pretreatment HCV-RNA viral load. In multivariate analysis, only pretreatment HCV-RNA less than 400 KIU/mL was associated with a sustained virological response (Table 3). The other factors, including zinc supplementation, BMI, staging, wk of HCV-RNA clearance, and pretreatment ALT level, did not influence sustained virological response.

Serum zinc concentrations

Serum zinc concentrations at 4 wk after the commencement of polaprezinc treatment were elevated and significantly higher than pretreatment levels in regimen A patients (Figure 2). Serum zinc concentrations in patients of regimen A were significantly higher than those of regimen B patients at 4 wk after the start of administration of polaprezinc (Figure 2).

Adverse effects

Each of the serious adverse events including depression, thrombocytopenia, leukopenia, and severe elevations in γ GTP was observed in one patient in regimen A. In addition, regimen B was also associated with serious adverse events such as depression in one patient, and severe leukopenia in two patients. Frequent adverse events and laboratory abnormalities recorded during the study are

Table 4 Incidence of selected adverse events (>10% frequency)

| Adverse event | Regimen A (CIFN + zinc) | Regimen B (CIFN) |
|--------------------|-------------------------|------------------|
| <i>n</i> | 41 | 42 |
| Fever | 41 (100%) | 42 (100%) |
| Headache | 17 (41.5%) | 16 (38.1%) |
| Malaise | 15 (36.6%) | 12 (28.6%) |
| Anorexia | 14 (34.1%) | 19 (45.2%) |
| Arthralgia | 14 (34.1%) | 13 (31.0%) |
| Insomnia | 11 (26.8%) | 11 (26.2%) |
| Stomach discomfort | 7 (17.1%) | 5 (11.9%) |
| Alopecia | 6 (14.6%) | 6 (14.3%) |
| depression | 6 (14.6%) | 5 (11.9%) |
| Nausea | 6 (14.6%) | 5 (11.9%) |
| Myalgia | 5 (12.2%) | 6 (14.3%) |
| Rash | 5 (12.2%) | 4 (9.5%) |

Table 5 Incidence of selected laboratory abnormalities (>10% frequency)

| Laboratory abnormality | Regimen A (CIFN + zinc) | Regimen B (CIFN) |
|---------------------------|-------------------------|------------------|
| <i>n</i> | 41 | 42 |
| Thrombocytopenia | 31 (78%) | 29 (69%) |
| Leucopenia | 24 (59%) | 30(71%) |
| Increase in AST | 20 (49%) | 18 (43%) |
| Increase in ALT | 18 (44%) | 16 (38%) |
| Increase in γ -GTP | 12 (29%) | 12 (29%) |
| Decrease in hemoglobin | 7 (17%) | 9 (21%) |
| Increase in ZTT | 7 (17%) | 7 (17%) |

summarized in Tables 4 and 5. The type and frequency of the adverse events, and laboratory abnormalities were similar in both regimens. Five (12.2%) patients and six (14.3%) patients dropped out of the study due to the adverse events before wk 24 in regimen A and regimen B, respectively. There were no significant differences of grading severity of adverse effects as well as incidence of them in the global safety assessment of the two regimens (data not shown).

DISCUSSION

The metabolism of trace elements is impaired in chronic liver disease^[19]. Zinc deficiency in chronic hepatitis C is improved by IFN treatment^[28]. Zinc supplementation enhances the response to IFN- α treatment in patients with genotype 1b chronic hepatitis C^[20]. Conversely, zinc supplementation did not enhance the response to CIFN treatment in patients with chronic hepatitis C with genotype 2 in this trial. The reasons for the lack of synergy between zinc and IFN are unclear, however, there are several possible reasons that are worthy of consideration. The viral genotype 2 in both regimens may account for the virological response, differing from the previous study that involved patients with genotype 1b. It is also possible

that the relatively higher response rate achieved with CIFN in both regimens in this trial compared with the previous study with IFN- α could have masked any differences in efficacy. In addition, individual differences in serum zinc concentration increases, as a result of zinc administration, may alter the virological response because the extent of the increases in serum zinc concentration varied in regimen A. Furthermore, differences in the type of IFN may affect the virological response, given that natural IFN- α was used in the previous study.

The overall therapeutic efficacy (60% SVR) of CIFN in our study was similar to that (50% SVR) of low-titer or genotype 2 group in a previous report from Japan^[18]. Recent reports showed that pegylated-IFN plus ribavirin^[29] had excellent treatment efficacy (100% SVR) in chronic hepatitis C patients with genotype 2^[30]. SVR rates of IFN- α 2b plus ribavirin for chronic hepatitis C patients with genotypes except 1^[11] or CIFN plus ribavirin for chronic hepatitis C patients with genotypes 2/3 were reported to be from 60% to 70%^[31]. Given the overall 60% SVR in our study, CIFN without ribavirin can be considered as a good candidate for patients in whom ribavirin is contraindicated.

Interestingly, the cases of undetectable HCV-RNA titer at 1 wk, but not 4 or 12 wk after the start of treatment, showed 95% and 100% of sustained virological response rate in regimens A and B, respectively. Although only a pretreatment HCV-RNA less than 400 KIU/mL was considered to be a predictive factor for sustained virological response, based on a logistic multiple regression model in our trial, earlier responses at 1 wk after the start of treatment may also be a good predictor of sustained virological response.

The type and frequency of adverse events, and laboratory abnormalities were similar in both regimens. Overall, adverse events and laboratory abnormalities were similar to those in previous reports in Japan^[18]. Although 18 MIU CIFN was relatively well tolerated in our trial, there were a few cases of serious adverse events. Twelve MIU and nine MIU CIFN, which are now available in Japan, may allow us to reduce the incidence of serious adverse events.

In conclusion, our study does not support the use of zinc as an adjunct to CIFN for the treatment of chronic hepatitis C with genotype 2, because combination treatment was not superior to CIFN monotherapy in achieving a sustained virological response. High viral loads of over 400 KIU/mL may indicate a low probability of sustained virological response for CIFN treatment, regardless of zinc supplementation.

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Absence of *MutY* homologue mutation in patients with multiple sporadic adenomatous polyps in Korea

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Abstract

AIM: Recently, germ-line mutation in the base excision repair gene *MYH* has been identified to cause a novel autosomal recessive form of familial adenomatous polyposis (FAP). Interestingly, a striking evidence for *MYH* mutations within different ethnic groups has been demonstrated. In this study, we screened 30 patients with multiple adenomatous polyps for *MYH* mutations to assess its prevalence and ethnic specificity in Korea.

METHODS: Thirty patients (21 men and 9 women; mean age 62.3 years) with multiple adenomatous polyps were examined for *MYH* mutations. The mean number of adenomas per patient was 10.0. Sixteen exonic regions and their intronic sequences were amplified by PCR and subjected to SSCP and DNA sequencing analyses.

RESULTS: None of the patients was identified to carry any truncating or sequence alterations in *MYH*. Our screening for the mutational regions, which were recognized from Caucasian patients or affected Indian families, also failed to detect sequence substitutions.

CONCLUSION: Mutation in *MYH* may be rarely involved in the pathogenesis of multiple sporadic colorectal adenomas in Korean population, although a large-scale analysis will be required to clarify the presence of specific *MYH* variants in a subset of patients and their role in the predisposition of multiple colorectal adenomas in Korean population.

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Key words: MY; Multiple adenomatous polyps; Germ-line mutation; Familial adenomatous polyposis; Ethnic difference

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INTRODUCTION

It has been well established that genetic factors play a pivotal role in up to 35% of all colorectal cancers (CRC)^[1-3]. Familial adenomatous polyposis (FAP) is an autosomal dominant disorder with an increased predisposition to multiple colorectal adenomatous polyps, and thence to CRCs^[4]. Classic FAP is caused by inherited mutations in the adenomatous polyposis coli (*APC*) gene, which encodes a protein that plays a critical role in the regulation of colonic cell growth^[5,6]. Attenuated FAP (AFAP) is associated with smaller numbers of adenomas and is caused by mutations in the extreme of 5' or 3' ends of *APC* or in the alternatively spliced region of exon 9^[4,5].

Somatic mutations of *APC* consist of the substitution of a thymine-adenine pair for a guanine-cytosine pair (G:C→T:A), which is a typical change caused by oxidative damage to DNA^[7-11]. Oxidative DNA damage produces the stable 8-oxo-7, 8-dihydro-2'-deoxyguanosine (8-oxoG)^[9]. It is highly mutagenic because it tends to mispair with adenine residues, leading to an increased frequency of spontaneous G:C→T:A transversion mutations in repair-deficient bacteria and yeast cells^[10-13].

Recently, Al-Tassan *et al*^[14] reported a novel autosomal recessive form of FAP. They found that three of seven siblings from a single British Caucasian family were all compound heterozygous for two non-conservative missense variants, Y165C and G382D, in the base excision repair gene *MutY* homologue (*MYH*). This finding first implicated a defect in the base excision repair mechanism in inherited predisposition to colorectal tumors in human beings.

Base excision repair (BER) is cell's mechanism of protection against oxidative DNA damage^[15]. The BER pathway repairs mutations caused by reactive oxygen species that are generated during aerobic metabolism^[16]. Three main components of BER have been identified in human. *MutT* homologue (*MTH*) removes oxidized base from 8-oxoG:C pairs to reduce the chances of incorporation of

Table 1 Characteristics of patients

| Characteristics | Patient with CRC and adenomatous polyp (n = 10) | Patient with adenomatous polyp only (n = 20) |
|--------------------------------------|---|--|
| Sex | 3 males : 1 females | 15 males : 5 females |
| Age (yr) | Mean 45.9 (range: 21 ~ 78) | Mean 51.8 (range: 44 ~ 69) |
| Number of adenomatous polyps | Mean 9 (range: 1 ~ 10) | Mean 7 (range: 3 ~ 100) |
| Family history of adenomatous polyps | 0 | 0 |

CRC: colorectal cancer.

8-oxoG during DNA replication, and 8-oxoG glycosylase (OGG) initiates base-excision of 8-oxoG from 8-oxoG:C pairs^[17]. Adenine-specific DNA glycosylase *MYH* removes adenine mispaired with 8-oxoG in 8-oxoG:A pairs.^[18-20]

The prevalence of *MYH* mutations has been determined in patients with multiple colorectal polyps in Caucasian populations. However, to our knowledge, a few studies have been carried out regarding the prevalence of *MYH* mutations in Asian patients with multiple colorectal adenomatous polyps. In the present study, we therefore explored the possible implication of germ-line mutations of *MYH* in the development of multiple sporadic colorectal adenomatous polyps in Korean population.

METHODS

Specimens

Thirty patients (21 men and 9 women; mean age, 62.3 years) with multiple sporadic colorectal adenomatous polyps were recruited from Kyung Hee University, Medical Center, Seoul, Korea. All patients were Korean and none had a family history of vertical transmission of colorectal cancer or adenomatous polyps. Five individuals who did not have any recognizable diseases were selected as healthy controls. Informed consent was taken from all patients. Ten milliliters of blood were obtained from all the patients and healthy controls for extraction of genomic DNA. The characteristics of patients are summarized in Table 1.

PCR amplification of *MYH* gene

Genomic DNA was prepared from venous blood samples using QIAGEN Genomic-tip system (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The concentration of extracted DNA was determined by a spectrophotometric measurement (Schimadzu Scientific Instruments, Inc., Concord, CA). Exons 1-16 of *MYH* and their flanking intronic sequences were amplified by PCR with 14 primer sets which cover the entire coding region of *MYH*. Primer sequences used for PCR amplification are shown in Table 2. PCR was performed with 200 ng genomic DNA as template for 38 cycles at 95°C (1 min), 58-62°C (30 s), and 72°C (30 s) in 1.5 mmol/L MgCl₂-containing reaction buffer (PCR buffer II, Perkin Elmer-Cetus, Concord, CA). Ten microliters of PCR products were electrophoresed on 20 g/L agarose gel containing ethidium bromide, visualized under ultraviolet light, and photographed.

Single strand conformation polymorphism analysis

To identify sequence alterations in the *MYH* gene, we

Table 2 Primer sequences of oligonucleotide primers used for PCR-SSCP analysis of *MYH*

| Exon | Primers | Sequences (5' to 3') | Length (bp) |
|------|---------|-------------------------|-------------|
| 1 | M1S | CAGAGCGCAGAGGCTTTGAACA | 240 |
| | M1AS | CTGAACGGAAGTTTCGACCCATC | |
| 2 | M2S | AATTTGGCCTCATTGTGACTGA | 221 |
| | M2AS | AATCTGCCTTTTCATGGCCAATG | |
| 3 | M3S | CACAGGCTGCTGTGCCAAGA | 259 |
| | M3AS | CCCACCCACTGTCCTGCTCCT | |
| 4+5 | M45S | AACTCCTCATCTGGGGTTGCAT | 296 |
| | M45AS | GGTCTGACCCATGACCCTTCCC | |
| 6+7 | M67S | ACCACCTTCACCTTGACCTTG | 268 |
| | M67AS | ACCCAAGACTCCTGGGTTCCCTA | |
| 8 | M8S | GGAACCCAGGAGTCTTGGGTGT | 222 |
| | M8AS | AAGGAGGCTGGGCACGCACAAA | |
| 9 | M9S | TTTGTGCGTGCCAGCCTGGTT | 223 |
| | M9AS | TGCTGTGAAGCAGAGCTCCAAA | |
| 10 | M10S | AAAGGAGCTCTGCTTCACAGCA | 227 |
| | M10AS | CACTCCTTAGGACTTCTCACTG | |
| 11 | M11S | GTAAGCCTACTGGGGAAGGGG | 222 |
| | M11AS | GCAGAATCTTACTCAGGTTAG | |
| 12 | M12S | GCCCTCTTGGCTTGAGTAGGGT | 278 |
| | M12AS | TCTCTTGTTACTCATGCCACTG | |
| 13 | M13S | AGGGAATCGGCAGCTGAGGCCCT | 226 |
| | M13AS | AAAAGCCAACATCCTTGGCTAT | |
| 14 | M14S | TATATCCACAGGCTATTGAA | 256 |
| | M14AS | ATATTCATGTAGAACATGTAGG | |
| 15 | M15S | GACATGAAGTTAAGGGCAGAAC | 212 |
| | M15AS | TGTTACCCAGACATTCGTTAG | |
| 16 | M16S | AACTACAAGGCCTCCTCCTTCCA | 270 |
| | M16AS | AACAACAGGATTCTCAGGGAATG | |

performed nonisotopic PCR-SSCP analysis as described previously^[14]. Briefly, 20 µL of the PCR products was mixed with 5 µL of 0.5 mol/L NaOH, 10 mmol/L EDTA and 10 µL of denaturing loading buffer (950 mL/L formamide, 20 mmol/L EDTA, 0.5 g/L bromophenol blue, and 0.5 g/L xylene cyanol). After heating at 95°C for 5 min, the samples were rapidly loaded in wells pre-cooled to 4°C and run simultaneously on two 80 g/L non-denaturing polyacrylamide gels with or without 100 g/L glycerol. These two gels were run at 18-20°C and then repeated at 6-10°C in a buffer-jacketed gel apparatus (DGGE-II; Aladin Enterprises, Inc., San Francisco, CA). Following an 8 h run at 400 volts, the gels were stained with ethidium bromide and photographed under ultraviolet light.

Automated DNA sequencing

PCR amplification products were purified using the PCR purification kit (Qiagen). DNA sequencing was carried out using ABI PRISM 377 automated DNA sequencer (Applied Biosystems, Foster City, CA) according to the

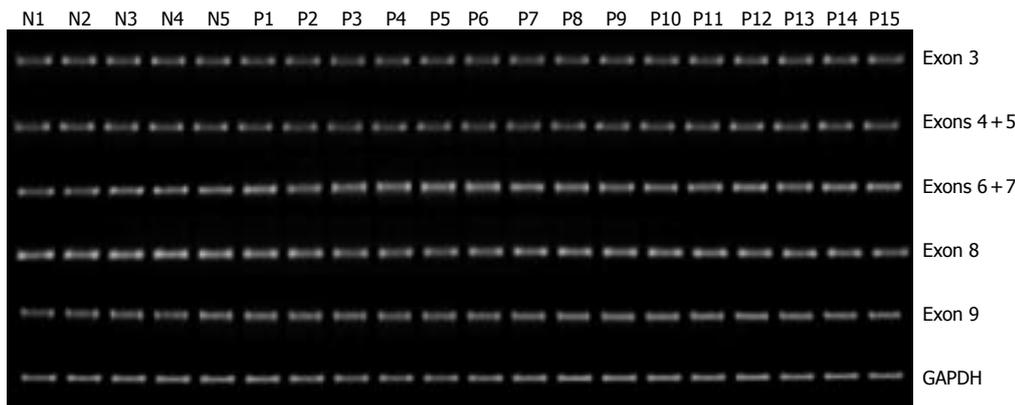


Figure 1 Genomic status of *MYH* in patients with multiple colorectal adenomas determined by quantitative DNA-PCR analysis. Representative examples for exons 3-9 amplification are shown. Two hundred nanograms of DNA obtained from 30 patients and 5 healthy controls were used as templates for PCR amplification of 16 exonic regions using 14 intronic primer sets. GAPDH was used as an internal control for PCR. Ten microliters of PCR product was resolved on 20 g/L agarose gel and visualized by ethidium bromide staining. No significant reduction of gene levels was identified in samples from patients as compared with normal controls. Lanes N1-N5: Normal controls; Lanes P1-P15: Patients.

manufacturer's instructions. Sequencing was carried out in both directions to confirm the findings.

RESULTS

To explore the presence of germ-line alterations of *MYH*, we, using quantitative genomic PCR analysis, initially evaluated genomic status of *MYH*. Blood DNA samples from 30 patients with multiple sporadic colorectal adenomatous polyps and 5 healthy individuals were subjected to PCR amplification of *MYH*. All of the 16 exons and flanking intronic sequences of the gene were amplified as 14 fragments using intron-specific primer sets, and their levels in the patients were compared with those in healthy controls. As shown in Figure 1, genomic levels of *MYH*, which were evaluated for 14 separate gene regions, showed no detectable difference between patients and healthy individuals, and none of the patients was found to have structural alteration within these protein-encoding regions of the gene. Although systemic analysis for the genomic status was not carried out in this study, the results suggested that our patients did not harbor germ-line deletion or structural abnormalities of the gene, which could result in loss or significant reduction of *MYH* protein function.

Using PCR-SSCP and DNA sequencing analyses, we next screened for mutations and sequence variants across the entire coding region of the *MYH* gene. To detect the *MYH* variants, such as Y165C, G382D, Y90X and E466X, which are commonly observed in Caucasian patients or affected Indian families, we performed comprehensive SSCP assay under four different running conditions^[14]. However, we could not observe any truncating *MYH* mutations in patient or control samples. Neither did we identify any likely pathogenic mutations and sequence variants in the patient samples. Representative examples of SSCP analysis are shown in Figure 2.

DISCUSSION

Germ-line mutation of the *MYH* gene has been known to

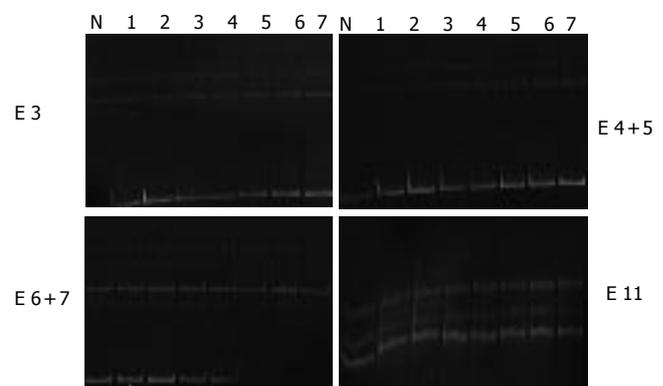


Figure 2 Non-isotopic PCR-SSCP analysis of *MYH*. For detection of sequence alterations, all of the 16 exons and flanking intronic sequences were amplified by PCR as 14 separate fragments, and 20 μ L of PCR product was subjected to non-isotopic SSCP analysis. Genomic DNA isolated from the healthy controls was used as normal controls. None of patient samples showed abnormal migration shift of single strand DNA molecules. Lane N: Normal control; Lanes 1-7: Patients; Lane E: Exons.

predispose persons in a variety of European populations to recessive inheritance of multiple colorectal adenomatous polyposis and classic FAP^[4, 22]. In addition, all patients with biallelic *MYH* mutations have an increased predisposition to CRCs.

MYH mutation presents with a distinguished genetic pathway in developing colorectal tumors. Many questions regarding its role in pathogenesis of multiple colorectal adenomatous polyps and CRCs still remain open. For example, it has not been identified why germ-line *MYH* mutations are associated with tumors of gastrointestinal tract, or why *MYH* polyposis differs in its phenotype and inheritance from classical FAP^[21].

It is difficult to distinguish between patients with *APC* mutations and those with *MYH* germ-line mutation on the basis of clinical features or pathological findings. *MYH* mutations seem to be a more common cause of the multiple adenoma phenotypes than are *APC* mutations^[23-26]. They are, however, a less common cause of classic colorectal adenomatous polyposis.

Jones *et al*^[4] could not identify any pathogenic variants in BER genes *OGG1* or *MTH1* in cases with colorectal tumors. Thus, there is a possibility that these genes are less frequently mutated than *MYH*. It is also possible that those mutations do not predispose to tumors in humans due to unknown mechanism. However, further studies are required to determine whether *OGG1* or *MTH1* are involved in CRC predisposition.

On the basis of previous studies, genetic analysis of *MYH* mutation should be considered for patients with a phenotype resembling FAP or AFAP, when no clear evidence of vertical transmission is noted. However, whether it is worthwhile to perform genetic testing in the family of patients with *MYH* mutations is a question that still remains unclear.

In the present study, we failed to detect any truncating or sequence alterations of the *MYH* gene in Korean patients with multiple colorectal adenomas. Although patient numbers enrolled in this study are too small to exclude the association of *MYH* mutations with development of multiple colonic adenoma in Korean population, our data suggests the presence of ethnic difference in contribution of *MYH* mutations to disease development between the Korean patients and the white patients. Previous studies identified four British families that were either homozygous for Y165C or compound heterozygous for Y165C/G382D, three Indian families that were homozygous for E466X, and a single Pakistani family that was homozygous for Y90X^[4]. Specific *MYH* mutations appear to be identified in different ethnic groups. A question still remains as to how frequently *MYH* mutations contribute to the phenotype of apparently sporadic AFAP/FAP. Further studies of patients from distinct geographical and ethnic groups will help to define the possible ethnic differences in actual mutations.

In conclusion, our observations suggest that *MYH* mutations are not common in patients with multiple colorectal adenomas in Korean population. Considering that number and histology of colorectal polyps are the cornerstones of detection of many CRC predisposition conditions, further larger-scale evaluation including the histologic analyses of the polyps, is required to determine whether *MYH* polymorphisms play a role in predisposition of a subset of multiple sporadic colorectal adenomas in Korean population.

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RAPID COMMUNICATION

Risk factors of acute cholecystitis after endoscopic common bile duct stone removal

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Abstract

AIM: To evaluate the risk factors of acute cholecystitis after endoscopic common bile duct (CBD) stone removal.

METHODS: A total 100 of patients who underwent endoscopic CBD stone removal with gallbladder (GB) *in situ* without subsequent cholecystectomy from January 2000 to July 2004 were evaluated retrospectively. The following factors were considered while evaluating risk factors for the development of acute cholecystitis: age, gender, serum bilirubin level, GB wall thickening, cystic duct patency, presence of a GB stone, CBD diameter, residual stone, lithotripsy, juxtapapillary diverticulum, presence of liver cirrhosis or diabetes mellitus, a presenting illness of cholangitis or pancreatitis, and procedure-related complications.

RESULTS: During a mean 18-mo follow-up, 28 (28%) patients developed biliary symptoms; 17 (17%) acute cholecystitis and 13 (13%) CBD stone recurrence. Of patients with acute cholecystitis, 15 (88.2%) received laparoscopic cholecystectomy and 2 (11.8%) open cholecystectomy. All recurrent CBD stones were successfully removed endoscopically. The mean time elapse to acute cholecystitis was 10.2 mo (1-37 mo) and that to recurrent CBD stone was 18.4 mo. Of the 17 patients who received cholecystectomy, 2 (11.8%) developed recurrent CBD stones after cholecystectomy. By multivariate analysis, a serum total bilirubin level of <1.3 mg/dL and a CBD diameter of <11 mm at the time of stone removal were found to predict the development of acute cholecystitis.

CONCLUSION: After CBD stone removal, there is no need for routine prophylactic cholecystectomy. However, patients without a dilated bile duct (<11 mm) and jaundice (<1.3 mg/dL) at the time of CBD stone removal

have a higher risk of acute cholecystitis and are possible candidates for prophylactic cholecystectomy.

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Key words: Sphincterotomy; Choledocholithiasis; Acute cholecystitis; Cholecystectomy

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INTRODUCTION

Endoscopic sphincterotomy is the treatment of choice for patients with common bile duct (CBD) stones. The high success rate and safety of this modality have been well established by a number of studies^[1-5]. Because gallstones may pass from the gallbladder (GB) into the CBD, calculous GB is considered to be one of the risk factors for the recurrence of bile duct stones after sphincterotomy^[4,6]. Some studies have compared the results of the wait-and-see policy and prophylactic cholecystectomy to prevent biliary complications but arrived at contradictory results^[7-9]. Thus, it still remains controversial as to whether subsequent laparoscopic cholecystectomy is indicated in patients with concurrent GB stones. Most studies have focused on the identification of predictors of CBD stone recurrence, which can be treated again endoscopically without surgery; however, the development of acute cholecystitis is a definite indication of cholecystectomy. So the risk factors of subsequent acute cholecystitis are more important than those of recurrent CBD stones in terms of the decision concerning prophylactic cholecystectomy. However, few studies have identified the risk factors of acute cholecystitis after endoscopic CBD stone removal in patients with GB *in situ*. The aims of this study were to assess the risks of biliary symptom recurrence and to identify the risk factors of acute cholecystitis in patients with GB *in situ* who have received endoscopic CBD stone removal.

MATERIALS AND METHODS

Patients

The medical records of patients with GB *in situ* who

Table 1 Baseline characteristics and CBD stone recurrence

| | Cholecystectomy due to acute cholecystitis | |
|-------------------------|--|---------------|
| | Yes (n=17) | No (n=83) |
| Mean age±SD | 58.9±16.3 | 65.4±12.0 |
| Gender (M/F) | 8/9 | 54/29 |
| Recurrence of CBD stone | 2/17 (11.8%) | 11/83 (13.2%) |

underwent endoscopic CBD stone removal for the first time in our hospital from January 2000 to July 2004 were reviewed. Patients were excluded if another neoplasm or acute cholecystitis was diagnosed at the time of CBD stone removal, and if they had undergone prophylactic cholecystectomy after CBD stone removal.

The diagnosis of CBD stone was made by either ultrasonography (USG) or computed tomography (CT) before endoscopic retrograde cholangiopancreatography (ERCP) in the majority of patients, but a definite diagnosis was defined as visible CBD stones on ERCP. The presence of GB stone was evaluated using USG, CT, and ERCP and the wall thickness of GB was evaluated using either USG or CT. Patients with no visible stone on all imaging modalities were allocated to the no GB stone group. The patients who required treatment for a CBD stone 6 mo after complete endoscopic stone removal were defined as having CBD stone recurrence. Radiological data were analyzed using a digitalized picture archiving communication system (PACS). Patient records were checked in each case to ascertain whether cholecystectomy due to acute cholecystitis was done or not after endoscopic CBD stone removal, and telephone calls were made to determine this in the few patients lost during follow-up.

Endoscopy protocol

Duodenal endoscopic intubation (TJF-240, Olympus, Tokyo, Japan) was performed under midazolam sedation. Sphincterotomy was performed in all the patients using a standard sphincterotome and/or a needle knife. After visualizing a CBD stone by cholangiography under fluoroscopic guidance, stones were extracted using a stone basket, balloon catheter, or a mechanical lithotripter according to stone size.

Statistical analysis

Data were analyzed using a statistical software package (SPSS, version 12.0; SPSS Inc.). Differences between the groups were analyzed using the χ^2 test. Logistic regression analysis was used to estimate odds ratios. The cumulative rate of acute cholecystitis requiring cholecystectomy was calculated using the Kaplan-Meier method.

RESULTS

Patient population

During the study, a total of 1 986 patients underwent ERCP at our hospital. The diagnosis of a CBD stone was made for 452 (22.8%) patients, and endoscopic CBD stone removal was performed successfully in 414 (91.6%)

patients. Of these, 198 (47.8%) had a GB *in situ* at the time of endoscopic CBD stone removal. Forty-seven (23.7%) patients had a previous history of endoscopic CBD stone removal more than once, and 38 patients (19.1%) received subsequent cholecystectomy for the management of acute cholecystitis. Another 3 (1.5%) patients who experienced incidental cholecystectomy during cancer surgery (2 for gastric cancer and 1 for hepatoma) and 12 (6.0%) patients with inadequate medical information were omitted from the analysis. The total number of study subjects subjected to analysis was 100. No prophylactic cholecystectomy was planned during the follow-up for these patients.

Recurrence of biliary complications after endoscopic CBD stone removal

During a mean 18-mo follow-up, 28 (28%) patients developed biliary symptoms; 17 (17%) with acute cholecystitis and 13 (13%) with CBD stone recurrence. No difference was observed between those who received cholecystectomy and those who did not receive cholecystectomy in terms of mean age or gender (Table 1). The mean time elapsed between original CBD stone removal and acute cholecystitis was 10.2 mo (1-37 mo). Of the 17 patients who received cholecystectomy, 2 patients (11.8%) developed recurrent CBD stones after cholecystectomy. The CBD stone recurrence rate in patients without acute cholecystitis was 13.2% (11/83). All cases of CBD stone recurrence were managed by endoscopic stone removal without surgery. The mean time elapsed (\pm SD) from endoscopic CBD stone removal to the development of acute cholecystitis was 10.2 (\pm 10.6) mo with a range of 1-37 mo. All the patients in whom acute cholecystitis developed received emergency or elective cholecystectomy. Laparoscopic cholecystectomy was performed for all the patients initially and in 2 of the 17 patients (11.8%) the operation was converted to open cholecystectomy. The mean follow-up time (\pm SD) of the patients in whom acute cholecystitis did not occur was 18.4 mo (\pm 9.8) and the range was 4-44 mo.

Risk factors for acute cholecystitis after endoscopic CBD stone removal

The results of univariate analysis of potential risk factors for the development of acute cholecystitis after endoscopic CBD stone removal are shown in Table 1. Of the 15 variables, total bilirubin <1.3 mg/dL, the presence of a GB stone and a CBD diameter <11 mm were found to be significant by univariate analysis ($P=0.01$, 0.02 , and 0.03 , respectively). However, multiple logistic regression analysis with forward selection and backward elimination identified only total bilirubin <1.3 mg/dL and CBD diameter <11 mm, and both variables reached statistical significance (Tables 2 and 3). Cumulative rates of acute cholecystitis requiring cholecystectomy, according to these two variables were calculated by the Kaplan-Meier method as shown in Figures 1 and 2.

DISCUSSION

The present study found that the overall recurrence rate of CBD stone after endoscopic CBD stone removal was 13%

Table 2 Univariate analysis of potential risk factors for the development of acute cholecystitis after endoscopic CBD stone removal

| Variable | n | Acute cholecystitis (%) | OR ¹ | 95%CI ² | P value |
|-------------------------|----|-------------------------|-----------------|--------------------|---------|
| Gender | | | | | |
| Male | 62 | 8 (12.9) | 1 | | |
| Female | 38 | 9 (23.7) | 2.10 | 0.73-6.01 | 0.13 |
| Age | | | | | |
| ≥60 yr | 74 | 11 (14.9) | 1 | | |
| <60 yr | 26 | 6 (23.1) | 1.72 | 0.56-5.24 | 0.25 |
| Total bilirubin | | | | | |
| ≥1.3 mg/dL | 67 | 7 (10.6) | 1 | | |
| <1.3 mg/dL | 33 | 10 (31.3) | 3.83 | 1.29-11.31 | 0.01 |
| GB wall thickening | | | | | |
| No | 77 | 13 (16.9) | 1 | | |
| Yes | 23 | 4 (17.4) | 1.04 | 0.30-3.56 | 0.59 |
| Patency of cystic duct | | | | | |
| No | 45 | 8 (17.8) | 1 | | |
| Yes | 55 | 9 (16.4) | 0.91 | 0.32-2.58 | 0.53 |
| Presence of GB stone | | | | | |
| No | 48 | 4 (8.3) | 1 | | |
| Yes | 52 | 13 (25.0) | 3.67 | 1.10-12.18 | 0.02 |
| CBD diameter | | | | | |
| ≥11.5 mm | 88 | 12 (13.6) | 1 | | |
| <11.5 mm | 12 | 5 (41.7) | 4.52 | 1.23-16.59 | 0.03 |
| Residual stone | | | | | |
| No | 91 | 15 (16.5) | 1 | | |
| Yes | 9 | 2 (22.2) | 1.45 | 0.27-7.66 | 0.48 |
| Lithotripsy | | | | | |
| Yes | 13 | 0 (0) | 1 | | |
| No | 87 | 17 (19.5) | 1.24 | 1.12-1.38 | 0.11 |
| Diverticulum | | | | | |
| No | 57 | 11 (19.3) | 1 | | |
| Yes | 43 | 6 (14.0) | 0.68 | 0.23-2.01 | 0.34 |
| Liver cirrhosis | | | | | |
| No | 92 | 15 (16.3) | 1 | | |
| Yes | 8 | 2 (25.0) | 1.71 | 0.32-9.30 | 0.41 |
| Diabetes mellitus | | | | | |
| No | 88 | 16 (18.2) | 1 | | |
| Yes | 12 | 1 (8.3) | 0.41 | 0.05-3.40 | 0.35 |
| Cholangitis at ERCP | | | | | |
| No | 71 | 14 (19.7) | 1 | | |
| Yes | 29 | 3 (10.3) | 0.47 | 0.12-1.78 | 0.20 |
| Pancreatitis at ERCP | | | | | |
| No | 88 | 15 (17.1) | 1 | | |
| Yes | 12 | 2 (16.7) | 0.97 | 0.19-4.90 | 0.67 |
| Complication after ERCP | | | | | |
| No | 95 | 16 (16.8) | 1 | | |
| Yes | 5 | 1 (20.0) | 1.23 | 0.13-11.79 | 0.61 |

¹Odds ratio; ²confidence interval.

(13/100), and that the frequency of acute cholecystitis after endoscopic CBD stone removal was 17% (17/100). Because two patients in whom acute cholecystitis developed also experienced CBD stone recurrence and there was no case of cholangitis or CBD stricture, the overall recurrence rate of biliary-related events during follow-up after endoscopic CBD stone removal was 28% (28/100), which is similar to the rates of 5-24% reported

Table 3 Multivariate analysis of potential risk factors for the development of acute cholecystitis after endoscopic CBD stone removal

| Variable | OR ¹ | 95%CI ² | P value |
|----------------------|-----------------|--------------------|---------|
| Total bilirubin | | | |
| <1.3 mg/dL | 4.62 | 1.39-15.33 | 0.01 |
| ≥1.3 mg/dL | 1 | | |
| CBD diameter | | | |
| <11.5 mm | 5.10 | 1.19-21.80 | 0.03 |
| ≥11.5 mm | 1 | | |
| Presence of GB stone | | | |
| Yes | 2.98 | 0.83-10.63 | 0.09 |
| No | 1 | | |

¹Odds ratio; ²confidence interval.

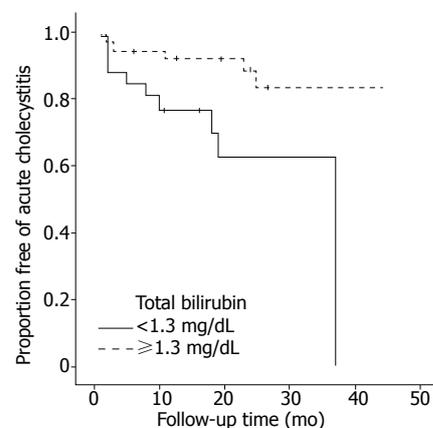


Figure 1 Kaplan-Meier's curve comparing two total bilirubin levels in relation to the development of acute cholecystitis. Patients with a total bilirubin of <1.3 mg/dL developed acute cholecystitis more frequently than patients with a level of ≥1.3 mg/dL ($P=0.01$, log-rank test).

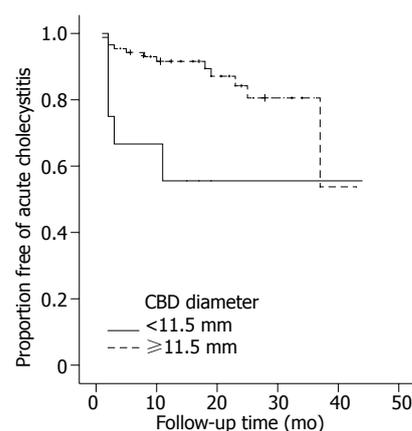


Figure 2 Kaplan-Meier's curve comparing two CBD diameters in relation to acute cholecystitis development. Patients with CBD diameters <11 mm developed acute cholecystitis more frequently than patients with CBD diameters of ≥11 mm ($P=0.03$, log-rank test).

previously^[1,2,4,6,8-28].

The advisability of prophylactic cholecystectomy to prevent biliary complications after endoscopic CBD stone removal remains a matter of debate; even

recent prospective^[7] and large scale cohort studies^[9] produced contrary conclusions concerning prophylactic cholecystectomy *vs* the wait-and-see approach. Moreover, because no consensus has been reached, the decision as to whether to operate or not is made empirically on a case-by-case basis^[8,13,17-19]. However, two important points should not be missed. The first is that, as presented by previous studies and ours, the occurrence of acute cholecystitis requiring cholecystectomy is not uncommon and the second is that morbidities such as bile duct injury and mortality caused by laparoscopic cholecystectomy are not negligible^[29-31]. Recurrent CBD stones can be retreated endoscopically without surgery but the development of acute cholecystitis is a definite indication for cholecystectomy. Thus the risk factors of subsequent acute cholecystitis are more important than that of recurrent CBD stones with respect to decisions concerning prophylactic cholecystectomy. In the present study, the development of acute cholecystitis (17%) was more common than that of CBD stone (13%) recurrence after CBD stone removal. We believed that if the risk factors of acute cholecystitis in patients who receive an endoscopic CBD stone removal could be identified, we might be able to identify the indicators of prophylactic cholecystectomy, as has been mentioned by earlier investigators^[13]. Some studies have compared the results of prophylactic cholecystectomy and non-surgical treatment^[7,9,17]. However, as far as we know, few studies have addressed the risk factors of acute cholecystitis after endoscopic CBD stone removal in patients with GB *in situ*, and thus our report might be the first to focus on this subject.

After conducting univariate and multivariate analyses to identify potential risk factors, we concluded that the occurrence of acute cholecystitis after endoscopic CBD stone removal was significantly more frequent in patients with a serum total bilirubin level of <1.3 mg/dL and a CBD diameter of <11 mm at the time of ERCP. Interestingly, a dilated CBD was identified as a risk factor of bile duct stone recurrence after endoscopic papillotomy by previous studies^[4,6]. However, our results should be interpreted from a different point of view because the pathogenesis of CBD stone recurrence and the development of acute cholecystitis differ. In East Asia the number of primary CBD stone cases is relatively high compared with the number of secondary CBD stones originating from a GB stone as compared with the situation in the West, although the number of secondary CBD stone cases is increasing over the recent years due to the Westernization of diet^[32-34]. In patients with GB or CBD stones, the differentiation of primary and secondary CBD stones is practically impossible because GB stones cannot be sampled for chemical analysis endoscopically, even though they are visualized by ERCP. However, considering the fact that patients with a dilated bile duct tend to form new stones in the bile duct and the cystic duct seldom passes large stones, large CBD stones with a dilated CBD are likely to be primary CBD stones^[4,26,35,36]. On the other hand, small CBD stones in association with a non-dilated CBD are more likely to originate from the GB. If cholecystectomy is not performed in such patients, acute cholecystitis development due to cystic duct obstruction

by a gallstone is a risk during the process of passage^[37,38]. The same assumption can be applied to patients with a low total bilirubin level who were found to develop acute cholecystitis more frequently. Of the 17 patients with cholecystectomy, 2 patients (11.8%) developed recurrent CBD stones after cholecystectomy. Thus cholecystectomy did not reduce CBD stone recurrence in our study, which suggests that most CBD stones may have been secondary rather than primary stones in our patients.

According to our data, 4 of 48 patients who were classified as acalculous at the time of ERCP developed acute cholecystitis during follow-up. A review of our operative records revealed that 2 of 4 patients had calculous cholecystitis and that the remaining 2 had no evidence of a GB stone. However, for the two acalculous cholecystitis patients, the possibility of calculous cholecystitis with a previously passed be excluded, because none of them had the well-known predisposing factors of acalculous cholecystitis, i.e., severe illness or old age^[39,40].

In conclusion, the present study shows that a serum bilirubin level of <1.3 mg/dL and a CBD diameter of <11 mm at the time of endoscopic CBD stone removal are risk factors for the development of acute cholecystitis requiring cholecystectomy. Although our results are limited due to the retrospective nature of our study, we suggest that prophylactic cholecystectomy should be reserved for patients who have the risk factors of acute cholecystitis. Prospective randomized studies are needed to more definitively establish the risk factors of acute cholecystitis after endoscopic CBD stone removal.

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Effect of interferon alpha and ribavirin treatment on serum levels of transforming growth factor- β 1, vascular endothelial growth factor, and basic fibroblast growth factor in patients with chronic hepatitis C

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Abstract

AIM: To assess the role of transforming growth factor- β 1 (TGF- β 1), vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) in the pathogenesis of fibrosis associated with chronic hepatitis C (CHC) and to evaluate the influence of the antiviral therapy on above parameter levels depending on the treatment results (complete response or no response).

METHODS: Study group included 100 patients with CHC, in whom fibrosis in liver specimens was assessed (Scheuer fibrosis score: 1-4 points). Control group included 30 subjects with antibodies anti-HCV present and persistently normal ALT level, without fibrosis (Scheuer fibrosis score: 0 points). Concentration of studied parameters was assayed in the serum by immunoenzymatic method before and after the therapy with interferon alpha-2b and ribavirin.

RESULTS: TGF- β 1 levels were significantly higher in the study group compared to the control group (35.89 vs 32.37 ng/mL; $P=0.023$). Such differences were not found in VEGF and bFGF levels. In patients showing complete response (negative HCV RNA and normal ALT level), significant increase in VEGF (112.8 vs 315.03 pg/mL; $P<0.05$) and bFGF (2.51 vs 15.79 pg/mL; $P=0.04$) levels were found. Significant decrease in TGF- β 1 level was observed both in responders (37.44 vs 30.02 ng/mL; $P=0.05$), and in non-responders (38.22 vs 30.43 ng/mL; $P=0.043$). bFGF levels before the treatment were significantly lower (2.51 vs 5.94 pg/mL; $P=0.04$), and after the treatment significantly higher (15.79 vs 4.35 pg/mL; $P=0.01$) in

patients with complete response than in those with no response.

CONCLUSION: Among the analyzed parameters TGF- β 1 seems to play the most important role in the pathogenesis of fibrosis in CHC. Levels of this factor are significantly lower in subjects who do not have fibrosis developed in them. Good therapeutic effect in CHC patients is associated with significant changes in TGF- β 1, VEGF, and bFGF levels. bFGF seems to have the highest usefulness in the prognosis of treatment efficacy.

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Key words: Liver fibrosis; HCV; Interferon; Ribavirin; Growth factor

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INTRODUCTION

Hepatitis C virus (HCV) is among the main causes of chronic liver diseases. Consequences of chronic HCV infection and high treatment costs make it a significant worldwide epidemiological, clinical, and social problem.

No pathological fibrosis is found during the initial stages of the disease, however in course of its development, the process increases, and the final stage is cirrhosis^[1]. Fibrosis as an after-effect of chronic liver inflammation is compared to wound healing^[2,3]. It is supposed that growth factors taking part in classical reaction of wound healing may play an important role in similar repair process occurring in the liver. Nature of fibrogenesis is excessive deposition of collagen and other extracellular matrix (ECM) components in the organ parenchyma. ECM protein synthesis takes place mainly in hepatic stellate cells^[2-4]. Inhibition and regression of fibrosis is connected with the limitation of excessive

ECM protein production and degradation of components already produced^[5,6]. Growth factors may be detected in blood that is circulated. There are premises that they could serve as markers of fibrosis and inflammatory activity, and could be useful in non-invasive hepatological diagnostics, especially in those situations when there are contraindications for liver biopsy^[2].

Among several known isomeric forms of transforming growth factor- β (TGF- β), the most significant role in pathogenesis of liver fibrosis is played by TGF- β 1. Main source of TGF- β 1 in the liver are activated stellate cells and Kupffer cells. The role of this factor in fibrosis development has been studied for several years^[5-10]. Results of those studies confirm the existence of a connection between the factor's concentration in the blood and progression of liver lesions^[8-10], as well as reduction of those concentrations following treatment leading to the decrease of fibrosis stage^[10].

Vascular endothelial growth factor (VEGF) stimulates angiogenesis both in regular conditions and in neoplastic processes^[11-15]. It has been verified to be closely involved in the development of hepatocellular carcinoma and hepatic metastases^[15].

Basic fibroblast growth factor (bFGF, FGF-2) is found in almost all tissues of mesoderm and endoderm origins. Studies conducted on animal models show participation of the cytokine in regeneration of tissues, angiogenesis and in wound healing process^[16,17]. A role played by bFGF in the processes of liver regeneration and fibrosis is not known. However, mentioned actions of the factor towards other tissues suggest possibility of its influence on fibrosis^[18].

The only way of liver fibrosis prevention known so far is effective causal treatment of hepatitis^[19]. Preferred treatment for patients with CHC is a combined therapy with interferon alpha and ribavirin. In antiviral treatment recombinant interferons alpha (alpha 2a and alpha 2b) are used, recently usage of pegylated interferons has become a standard. Ribavirin is an analog of guanosine. Apart from producing a virostatic effect, the drug shows probably also immunomodulative activity. Aim of CHC treatment is inhibition of HCV replication shown by the disappearance of HCV RNA in peripheral blood, which may lead to the decrease of inflammatory activity and stage of fibrosis found in histopathological examination.

We undertook this study to assess the role of TGF- β 1, VEGF, and bFGF in the pathogenesis of liver fibrosis, evaluate effect of antiviral treatment on concentration of these factors depending on the effect of the treatment (full response or lack of response), and define a biochemical marker useful for prognosis of antiviral treatment efficacy.

MATERIALS AND METHODS

Patients

Ethical approval for the study was obtained from the Bioethical Committee of Medical University of Silesia. All serum and tissue samples were taken with patients' informed consent.

Study group included 100 patients (48 females and 52 males), aged between 19 and 64 (average 42 ± 3 years) with chronic hepatitis C (CHC).

The diagnosis of CHC was confirmed by the presence of serum HCV-RNA assayed with reverse transcription polymerase chain reaction (RT-PCR) method (Amplicor v.2 Roche Diagnostic test) and persistently elevated alanine aminotransferase (ALT) level for at least 6 mo. All studied patients had liver biopsy performed with Hepafix kit (B. Braun, Melsungen AG, Germany). Grade of inflammatory activity and stage of fibrosis were assessed according to Scheuer numeric scale^[20]. Patients with pathologically confirmed cirrhosis met the criteria for the A class in Child-Pugh scale.

Control group included 30 patients with present anti-HCV antibodies and normal ALT activity, with no or minimal necroinflammatory activity and no pathological fibrosis found in liver specimen pathological examination.

Methods

All patients starting the therapy and those belonging to the control group had blood collected in the beginning of the study. Blood samples were also taken from the study group subjects after the completion of therapy. Obtained serum was frozen at -70°C until assay.

TGF- β 1, VEGF, and bFGF concentrations were measured by immunoenzymatic method (Quantikine Immunoassay, R&D Systems Inc, Minneapolis, USA).

In therapy of CHC patients interferon alpha 2b and ribavirin were used (RebetronTM, Schering-Plough). Interferon alpha was administered at 3 MU dose thrice a week, ribavirin at a dose of 1 200 mg/d for patients ≥ 75 kg of body weight, or at a dose of 1 000 mg/day for patients with body weight lower than 75 kg. Treatment was continued for 48 wk.

Serum levels of TGF- β 1, VEGF, and bFGF were measured before and after the treatment.

In this study, comparison was made between the two groups of patients - those with complete sustained response to applied antiviral treatment (undetectable HCV RNA and normalization of ALT activity 6 mo after the end of therapy) and lack of this response (continuous elevated aminotransferase activity and presence of HCV RNA after conclusion of therapy).

Statistical analysis

Values were expressed as the mean \pm SE. Hypothesis of studied parameters, mean values equality between the compared groups were assessed using Student's *t* test for related and unrelated variables preceded with Fisher's test for variance homogeneity and Mann-Whitney *U* test. $P < 0.05$ was considered statistically significant.

RESULTS

General characteristics of patients with CHC and subjects belonging to the control group are presented in Table 1.

Figure 1 shows the results of TGF- β 1 assays in compared groups. In group of patients with CHC, there was significantly higher serum concentration of the factor compared to control group (35.89 ± 1.28 vs 32.37 ± 1.41 ng/mL; $P = 0.023$).

VEGF serum levels in compared groups are demonstrated in Figure 2. No significant differences of

Table 1 Characteristics of patients

| | <i>n</i> | Male/female | Age (yr) | ALT (U/L) | AST (U/L) | Alkaline phosphatase (U/L) | Bilirubin (μ mol/L) |
|---------|----------|-------------|---------------|-------------------|-----------------|----------------------------|--------------------------|
| CHC | 100 | 52/48 | 42.08 \pm 3 | 108.7 \pm 10.12 | 75.6 \pm 8.76 | 141.7 \pm 6.78 | 18.9 \pm 2.42 |
| Control | 30 | 16/14 | 40.79 \pm 2 | 21.3 \pm 1.92 | 18.5 \pm 1.73 | 136.4 \pm 6.56 | 13.8 \pm 1.76 |

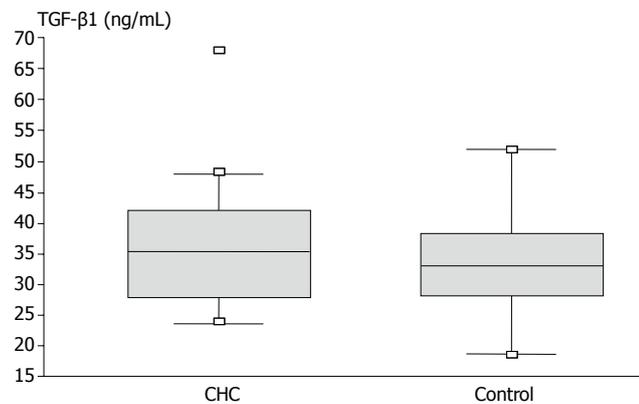


Figure 1 Serum levels of TGF- β 1 (ng/mL) in CHC group and control group. The box represents the interquartile range. The whiskers indicate the highest and lowest values. The line across the box indicates the median value.

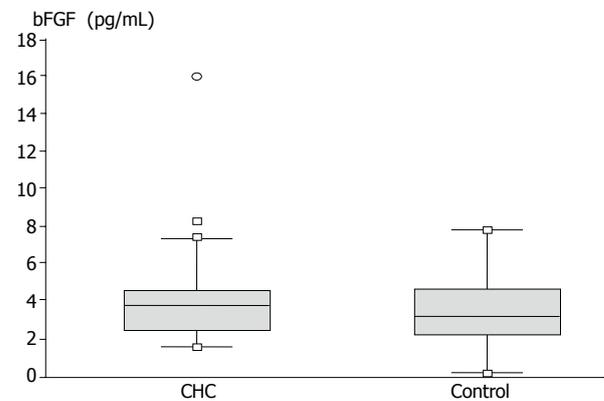


Figure 3 Serum levels of bFGF (pg/mL) in CHC group and control group. The box represents the interquartile range. The whiskers indicate the highest and lowest values. The line across the box indicates the median value.

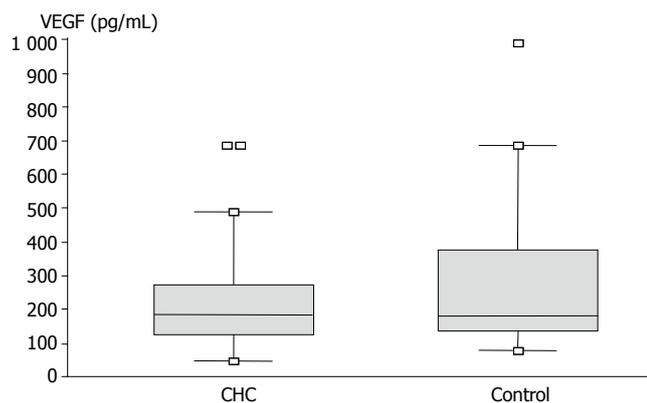


Figure 2 Serum levels of VEGF (pg/mL) in CHC group and control group. The box represents the interquartile range. The whiskers indicate the highest and lowest values. The line across the box indicates the median value.

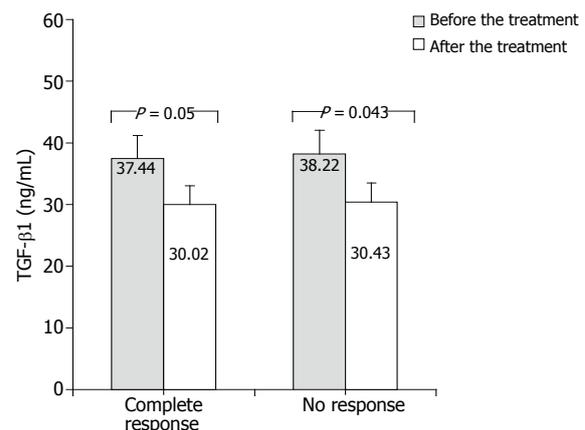


Figure 4 Comparison of TGF- β 1 levels (mean and SE) before and after the antiviral therapy of CHC patients depending on treatment efficacy.

this growth factor concentration were found between the hepatitis C group and control (231.84 ± 24.55 *vs* 262.89 ± 33.99 pg/mL). Figure 3 presents bFGF concentration. No significant differences were found between CHC group and control (4.35 ± 0.58 *vs* 3.30 ± 0.39 pg/mL).

In Figure 4, TGF- β 1 concentrations measured before and after antiviral treatment are compared. In both groups – responders and non-responders – significant decrease in serum TGF- β 1 concentration was found following antiviral treatment. TGF- β 1 level in responders decreases from 37.44 ± 4.31 to 30.02 ± 4.16 ng/mL ($P=0.05$) and in non-responders from 38.22 ± 3.93 to 30.34 ± 3.7 ng/mL ($P=0.043$). Figure 5 shows changes of VEGF concentration following treatment with interferon and ribavirin. In group of responders, significant increase of this factor concentration in blood was found (112.8 ± 10.2

vs 315.03 ± 29.3 pg/mL; $P<0.05$), unlike the group of non-responders. In Figure 6, concentrations of bFGF in treated patients are shown. Significant increase of the concentration was demonstrated in patients with complete response to therapy (2.51 ± 0.29 *vs* 15.79 ± 1.8 pg/mL; $P=0.04$). The concentration did not change significantly in the group of non-responders.

bFGF levels before the treatment were significantly lower (2.51 *vs* 5.94 pg/mL; $P=0.04$), and after the treatment significantly higher (15.79 *vs* 4.35 pg/mL; $P=0.01$) in patients with complete response than in those with no response.

DISCUSSION

Process of liver fibrosis has been recently a subject of numerous studies. Attempts are made to recognize

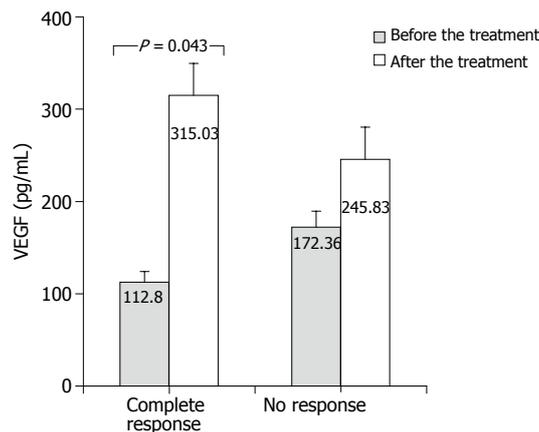


Figure 5 Comparison of VEGF levels (mean and SE) before and after the antiviral therapy of CHC patients depending on treatment efficacy.

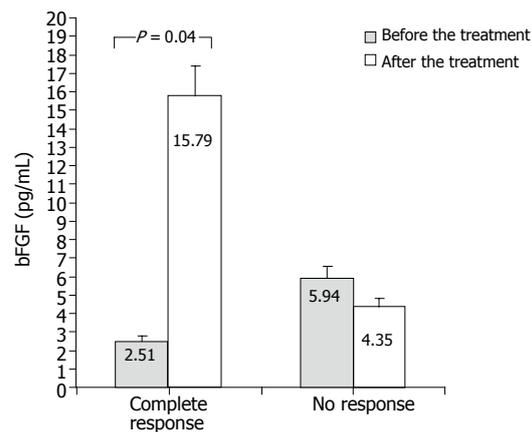


Figure 6 Comparison of bFGF levels (mean and SE) before and after the antiviral therapy of CHC patients depending on treatment efficacy.

accompanying pathogenetic phenomena and define factors influencing its progression. Reports were published concerning the possibility of fibrosis stage decrease following effective causal treatment, for example, following treatment of CHC with interferon and ribavirin^[6,10,21,22]. Some authors have reported even cases of regression of cirrhosis that has been considered irreversible so far^[2,6,10,19,23-26]. This study made an attempt to define the role that may be played by chosen growth factors in processes occurring in course of chronic hepatitis.

TGF- β 1 is the growth factor of the best known and confirmed pro-fibrogenic activity. In our study it was demonstrated that TGF- β 1 concentration in patients with fibrosis in course of CHC was indeed higher compared to the patients in whom there was no fibrosis development. Tsushima *et al* noted significant decrease of TGF- β 1 concentration in serum, and inhibited expression of TGF- β 1 mRNA in hepatic tissue following antiviral treatment, regardless of the clinical result (both for responders and non-responders)^[10]. Similar observations have been made during our study. For both groups of patients significant decrease of the factor concentration was noted, regardless of the final result of treatment. This could suggest existence of direct, inhibitory influence of interferon alpha and/or ribavirin on the expression of TGF- β 1 that is independent of the influence of the drug on HCV replication.

VEGF, as a factor specifically inducing proliferation of vascular endothelium cells, plays an important role in angiogenesis, and seems to play an indirect role in regeneration processes. One of the repair processes in which VEGF plays its role is probably "wound healing" process taking place in liver, and being a consequence of chronic inflammatory condition. Studies including analyses of VEGF participation in the development of hepatocellular carcinoma were performed^[11-15], but only single attempts to assess its blood concentration and hepatic expression in hepatitis have been reported. Akiyoshi *et al*^[27] found significant increase of VEGF concentration in the serum of patients with acute hepatitis of typical course, but in cases of fulminant hepatitis those concentrations were lower than in control group

and increased in patients who recovered. Low VEGF concentrations were noted by the authors in cases of cirrhosis, but they did not show significant differences compared to control group in cases of chronic hepatitis. Jinno *et al* obtained different results. In their study VEGF concentrations appeared to be significantly higher in cases of chronic hepatitis and cirrhosis compared to healthy individuals^[28]. In our study we did not find statistically significant differences of VEGF concentrations in blood between patients with hepatitis C and control group. No papers estimating possible changes in the cytokine concentration following antiviral therapy have been published yet. Our study showed that in patients meeting the criteria of response to this type of treatment there was a significant increase of VEGF serum concentration. The phenomenon was not observed in non-responsive patients. VEGF concentration increase following effective therapy, after which one could expect inhibition of fibrosis, justifies the assumption that stimulation of angiogenesis by this factor may be a key element in regeneration processes accompanying regression of liver fibrosis.

bFGF, a growth factor with well documented participation in classic process of "wound healing", may also play a role in repair processes taking place in liver damaged by inflammatory condition, however, number of published reports on this topic is very low. Jinno *et al* found that bFGF blood concentrations increase with the liver disease progression^[18]. In our study bFGF levels were not statistically different in case and control groups. Following treatment with interferon and ribavirin, significant increase of the factor concentration was found in patients, for whom the therapy proved to be effective. Moreover, it was noted that complete therapeutic response occurred in patients whose initial concentration of this cytokine was significantly lower, and significantly higher following the treatment than in non-responders group. As one of the probable bFGF activity courses is stimulation of endothelium cells proliferation^[16,17], it may be assumed that increase of the cytokine concentration following effective treatment may be linked with intensification of angiogenesis, just like in the case of VEGF.

Studies showed that antiviral treatment, especially effective one, causes significant changes in concentrations of some growth factors. Studies documenting decrease of fibrosis stage following the therapy and concluded with persistent inhibition of virus replication are increasing. Combination of those facts justifies assumption that those cytokines may play an important role in pathogenesis of changes taking place in liver during chronic inflammatory conditions. Studies documenting participation of the growth factors other than TGF- β 1 in those processes are, as for now, very sparse. It seems, however, that the topic deserves interest. Precise knowledge concerning processes leading to the development of fibrosis could contribute to elaboration of methods aimed at prevention of the disease progression and complications.

Among the factors analyzed in our study, TGF- β 1 plays the most significant role in pathogenesis of fibrosis in course of CHC. It supports results of other studies.

We have demonstrated that good therapeutic effect in patients with CHC is connected with influence of drugs on TGF- β 1, VEGF, and bFGF serum concentration. These findings suggest possible usefulness of these factors in assessment of antiviral treatment efficacy and indication of medication failure. It seems that bFGF shows the best prognostic value regarding final antiviral treatment results.

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RAPID COMMUNICATION

Conversion of cadherin isoforms in cultured human gastric carcinoma cells

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Abstract

AIM: To explore the expression of cadherin isoforms in cultured human gastric carcinoma cells and its regulation.

METHODS: The expressions of cell adhesion molecules (including E-cadherin, N-cadherin, α -catenin, β -catenin) and cadherin transcription factors including snail, slug and twist were determined by reverse transcriptase-polymerase chain reaction (RT-PCR), immunoblotting and immunofluorescence in SV40-immortalized human gastric cell line Ges-1 and human gastric cancer cell lines MGC-803, BGC-823 and SGC-7901.

RESULTS: All cell lines expressed N-cadherin, but not E-cadherin. N-cadherin immunofluorescence was detected at cell membranous adherents junctions where co-localization with immunofluorescent staining of inner surface adhesion proteins α - and β -catenins was observed. The transformed Ges-1 and gastric cancer cell lines all expressed transcription factors (snail, slug and twist) which inhibited the expression of E-cadherin and triggered epithelial-mesenchymal transformation.

CONCLUSION: Cadherin isoforms can change from E-cadherin to N-cadherin in transformed human gastric cancer cells, which is associated with intracellular events of stomach carcinogenesis and high expression of corresponding transcription factors.

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Key words: E-cadherin; N-cadherin; Transcription factor; Gastric cancer

Wang BJ, Zhang ZQ, Ke Y. Conversion of cadherin isoforms in cultured human gastric carcinoma cells. *World J*

INTRODUCTION

Cadherins are a family of transmembranous glycoproteins responsible for calcium-dependent intercellular adhesion, and play a dual role as adhesion ligands and receptors in cell adherents junctions (AJs). They are divided into more than ten subclasses, which are distinct in immunogenic specificities and tissue distribution. AJs are cell-to-cell adhesion sites where classic cadherins, cytoplasmic catenins (α -, β -, γ -) and actin-based cytoskeleton are assembled. Such protein complex plays a role as a functional unit in mediating cell adhesion and signal transduction and is involved in regulation of cell recognition, migration and cell sorting behavior^[2,3]. E-cadherin and N-cadherin, members of cadherin superfamily, are the most popular adhesion receptors in tissues. E-cadherin is predominantly expressed in epithelia, and loss of E-cadherin expression is a major characteristic of highly invasive and metastatic cancer^[4], while re-expression of E-cadherin results in reversion of metastatic phenotypes to benign phenotypes. E-cadherin has been known as a tumor suppressor against invasion and metastasis of tumors^[5]. N-cadherin is expressed in mesenchymal cells, but recent study demonstrated that N-cadherin is associated with an increased invasive potential of cancer^[6]. Forced expression of N-cadherin exerts a dominant effect over E-cadherin function in breast cancer cells^[7].

In early embryonic development, loss of E-cadherin accompanying the acquisition of fibroblastic phenotype occurs through a mechanism called epithelial-mesenchymal transition (EMT). This is an essential event during gastrulation and neural crest formation^[8] as well as in early invasion and metastasis of carcinoma cells^[9]. Multiple mechanisms can inactivate E-cadherin in cancer cells, such as gene mutation, promoter hypermethylation, chromatin rearrangement, and transcriptional repressors. Several developmentally important genes inducing EMT act as E-cadherin repressors. In vertebrates, snail and its closely related gene slug bind directly to E-boxes in the *E-cadherin* promoter and repress *E-cadherin* expression directly in mouse and human invasive carcinoma cells^[10]. A further molecule known to trigger EMT is twist, which is possibly involved in E-cadherin and N-cadherin conversion during

Table 1 PCR Primers used in experiments

| Genes | Primers (sense/anti-sense) |
|--------------------|---|
| E-cadherin (361bp) | 5'-TCCATTCTTGGTCTACGCC-3' 5'-TTTGTCTACCGACTTCCAC-3' |
| N-cadherin (373bp) | 5'-GTGCCATTAGCCAAGGGAATTCAGC-3' 5'-GGAGATACTCACCTTGTCTTGC-3' |
| Snail(377bp) | 5'-CTGCAGGACTTAATCCAG-3' 5'-CGAGAGACTCCGGTTCCTA-3' |
| slug(410bp) | 5'-AGCGAACTGGACACACATAC-3' 5'-TCTAGACTGGCATCGCAG-3' |
| twist(612bp) | 5'-GCAAGCTTAGAGATGATGCAGGACG-3' 5'-GACTCGAGGTGGGACGCGGACATGGA-3' |
| GAPDH (451bp) | 5'-ACCACAGTCCATGCCATCAC-3' 5'-ATGTCGTTGTCCACCACCT-3' |

EMT^[11]. In the present study, we chose a transformed human gastric cell line as control and three human gastric cancer cell lines to detect the expression of E-cadherin and N-cadherin subtypes and their associated adhesion protein (α - and β -catenins) related to changes of transcription factors (snail, slug and twist), and to analyze the role of snail and other EMT regulators in down-regulating E-cadherin in gastric cancer cells.

MATERIALS AND METHODS

Cell culture

Human gastric cancer cell lines MGC-803, BGC-823 and SGC-7901 were cultured in RPMI1640 medium (GIBCO/BRL) containing 10% fetal bovine serum. Ges-1, a non-tumorigenic but immortalized human gastric cell line, was obtained by SV40 viral transformation and cultured in RPMI1640 medium supplemented with 15% fetal bovine serum^[12].

Immunoblotting experiments

Cells of logarithmic growth potential treated with RIPA lysis buffer (50mmol/L Tris-HCl pH 7.4, 150mmol/L NaCl, 1% NP40, 50mmol/L NaF, 0.1mmol/L Na₃VO₄, 1mmol/L phenylmethylsulfonyl fluoride, 10 μ g/mL leupeptin, 0.1% SDS, 0.5% deoxycholic acid, 50mmol/L HEPES, 10mmol/L EDTA, 50mmol/L sodium pyrophosphate, 50mmol/L ammonium molybdate) were used to determine the total protein concentration according to Bradford protein quantification method. Samples of equal amount were loaded onto 10% SDS-PAGE gel and electrophoretically transferred to PVDF membrane, blocked with 5% non-fat milk/TTBS, reacted with primary antibody, washed with TTBS and labeled with goat and anti-mouse or rabbit secondary antibody. The primary antibodies used were mouse anti-N-cadherin monoclonal antibody, rabbit anti-E-cadherin polyclonal antibody, rabbit anti- α - and β -catenin polyclonal antibody (Sigma). Protein signals were detected using ECL chemiluminescence reagent (Amersham Pharmacia).

Indirect immunofluorescent staining

Cells growing on coverslips were fixed in 2% formaldehyde for 3 min and extracted 3 times (10 min each) with 0.5% Triton X-100. The cell samples were incubated with primary antibodies (described above) at 37°C for 1 h,

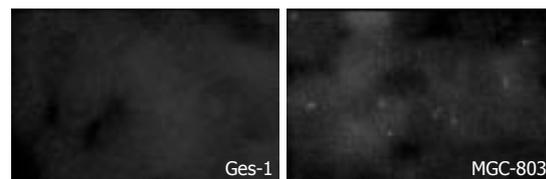


Figure 1 Expression of E-cadherin in SV40-immortalized Ges-1 and gastric cancer cell lines. Negative staining of E-cadherin in Ges-1 and MGC-803 was shown on immunofluorescent micrographs.

washed with PBS containing 0.5% Triton X-100 and then reacted with FITC- or rhodamine-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) at 37°C for 1 h. After washed, the samples were rinsed in 0.9% NaCl, stained with 1 μ g/L 4, 6-diamidino-2-phenylindole (DAP; Sigma) and mounted in 60% glycerol/PBS. Olympus BH2 fluorescent microscope equipped with FITC- and rhodamine- channel filter systems was used to observe distribution of different proteins in the same fields. Kodak TMAX-400 black and white films were used for photographs.

RT-PCR

Total RNA was extracted from cells of logarithmic growth potential with Trizol (Invitrogen) as described, then suspended in 50 μ L RNase-free water and quantified. Reverse-transcription was performed in a final volume of 20 μ L using superscript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions under the following conditions: 1mmol/L dNTP, 50ng Oligo-dT₁₅ and 3 μ g total RNA. The reaction was performed at 50°C for 45 min, followed by inactivation of the enzyme at 70°C for 15 min. The sequences of the specific primers are listed in Table 1. Forty cycles of PCR amplification were performed at 94°C for 1 min, at 58°C for 1 min, at 72°C for 1 min. PCR products were identified by running on 1.2% agarose gel.

RESULTS

E-cadherin expression lost in SV40-immortalized Ges-1 and gastric cancer cell lines

The three gastric cancer cell lines MGC-803, BGC-823, and SGC-7901 were negative for E-cadherin expression as demonstrated by RT-PCR, immunoblotting and immunofluorescent staining. The SV40-transformed gastric epithelial cell line Ges-1, non-tumorigenic in nude mice with normal cell morphology, was also negative for E-cadherin expression at mRNA and protein levels (Figure 1).

N-cadherin expression in SV40-immortalized Ges-1 and gastric cancer cell lines

In order to understand the formation of adherens junctions in these E-cadherin negative cell lines, N-cadherin expression was detected by RT-PCR, immunoblotting and

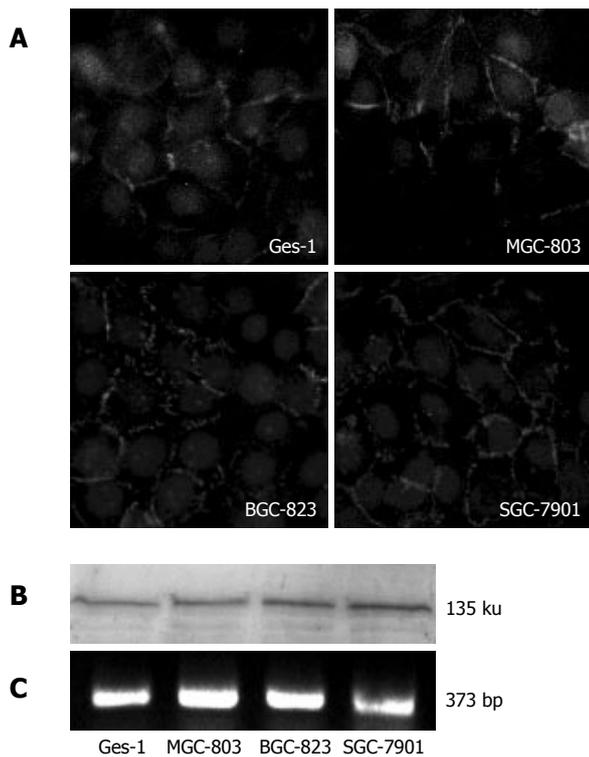


Figure 2 Expression of N-cadherin in SV40-immortalized Ges-1 and gastric cancer cell lines. **A:** Distribution of N-cadherin fluorescence on cell membranes in Ges-1 and gastric cancer cell lines; **B:** A band of 135 ku N-cadherin protein detected by Western blotting of N-cadherin; **C:** N-cadherin mRNA expression shown by RT-PCR.

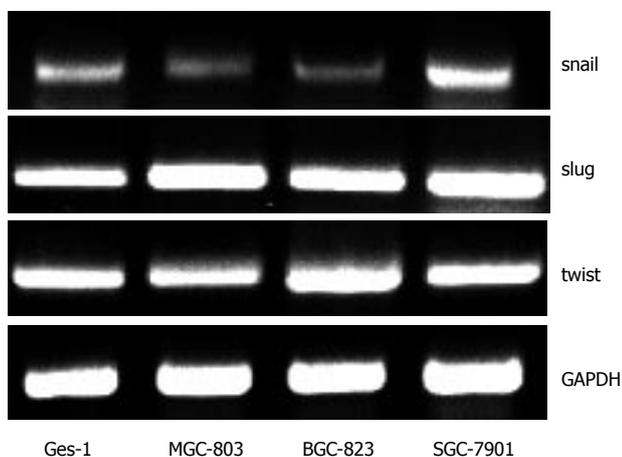


Figure 4 Expression of E-cadherin transcription repressors (snail, slug) and N-cadherin activator (twist) in Ges-1 and gastric cancer cell lines.

immunofluorescent staining (Figure 2). N-cadherin mRNA was found in all these E-cadherin negative cell lines (SV40-transformed Ges-1, BGC-823, MGC-803, and SGC-7901). In consistency with N-cadherin mRNA expression, a band of 135 ku N-cadherin protein was also shown in these cell lines as detected by Western blotting of N-cadherin. N-cadherin fluorescence was distributed at cell-cell contact membrane regions, suggesting that these cells established adherents junctions through N-cadherin rather than E-cadherin. Alteration of cadherin isoforms from E-cadherin to N-cadherin occurred in the process of carcinogenesis.

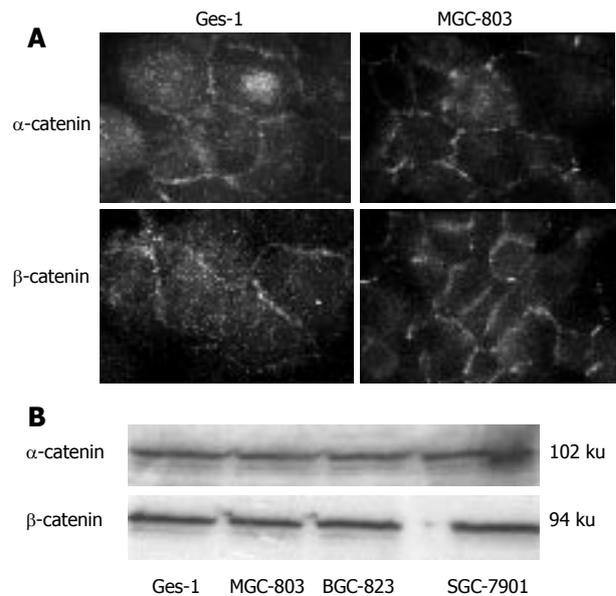


Figure 3 Expression of α - and β -catenins in Ges-1 and gastric cancer cell lines. **A:** Distribution of α - and β -catenin fluorescence on cell membranes and cell-cell borders in Ges-1 and MGC-803; **B:** Reactive bands at 102 ku and 94 ku of α - and β -catenins in Ges-1 and cancer cell lines.

Expression of α - and β -catenins in SV40-immortalized Ges-1 and gastric cancer cell lines

Immunoblotting of α - and β -catenins showed reactive bands at 102 ku and 94 ku respectively in Ges-1 and gastric cancer cell lines. The fluorescence of α - and β -catenins was found at the cell-cell contact borders irrespective of transformed cells or cancer cells (Figure 3). The colocalization of both cadherin and catenins indicated the formation of cadherin/catenin complex at adherents junctions.

Expression of E-cadherin transcription repressor and N-cadherin activator in Ges-1 and gastric cancer cell lines

The transcription factors (snail, slug and twist) could repress the expression of E-cadherin and induce the expression of N-cadherin. Thus, we examined the expression of these transcription factors in these cell lines with alteration of E-cadherin to N-cadherin by RT-PCR. They were all expressed in Ges-1 and three cancer cell lines, but their expression levels varied (Figure 4).

DISCUSSION

It is well known that E-cadherin is an adhesion receptor of normal epithelial cells that binds to cytoplasmic adhesion proteins to form E-cadherin/catenin complex as an anchorage site for actin-based cytoskeleton. The E-cadherin/catenin/cytoskeleton complex plays an important role in the maintenance of structural and functional stability of epithelial tissues. Loss of E-cadherin expression or function in epithelial carcinoma has been considered as a primary reason for disruption of cell-cell contacts. However, there is evidence that other cadherins, such as N-cadherin, are up-regulated in cancer cell lines^[13]. N-cadherin is present in invasive cancer cell lines and its exogenous expression in tumor cells increases the ability

of invasion and metastasis of tumor cells. Changes in the expression of cadherins occur in the early embryonic development during gastrulation and neural crest formation. The loss of E-cadherin with the acquisition of fibroblastic phenotype occurs through a mechanism called epithelial-mesenchymal transition (EMT) during early invasion and metastasis of carcinoma cells. To investigate the occurrence of cadherin conversion in gastric carcinoma, we determined the expression of E-cadherin and N-cadherin in transformed human gastric cell line and three gastric cancer cell lines. The results demonstrated that three human gastric cancer cell lines were all negative for E-cadherin immunofluorescent staining and immunoblotting, which is in agreement with the reported data, suggesting that E-cadherin expression is inhibited in gastric cancer cells^[14]. Meanwhile, our finding is in accordance with the report that loss of E-cadherin is a key step in the progression from benign adenoma to malignant carcinoma *in vivo*^[15]. However, in our study, N-cadherin was expressed in all these E-cadherin negative cell lines and N-cadherin fluorescence was distributed at cell-cell contact membrane regions, suggesting that these cells are established at the adherens junctions through N-cadherin rather than E-cadherin. Apparently, alteration of cadherin isoforms from E-cadherin to N-cadherin occurs in the process of carcinogenesis. SV40-transformed gastric epithelial cell line Ges-1 characterized by immortalization *in vitro* and normal cell morphology, was also negative for E-cadherin expression at mRNA and protein levels, suggesting that inhibition of E-cadherin expression may be an early cellular event in the process of cell malignancy.

The interaction between cytoplasmic cadherins and catenins is critical for the formation of stable and functional AJs. The mutation or homozygous deletion of catenin gene leads to loss of cell-cell adhesion. Moreover, β -catenin can act as a transcription factor in nuclei as an activator of LEF/TCF family of the DNA-binding proteins, which is activated by the Wnt signal pathway^[16]. We therefore investigated the expression of α - and β -catenins in the same cell lines. Immunoblotting and immunofluorescence staining showed positive expressions of α - and β -catenins in transformed Ges-1 and three cancer cell lines. Meanwhile, we did not find the relocalization of β -catenin in nuclei, suggesting that cadherin/catenin complex is formed at adherens junctions. In gastric cancer cells, the formation of N-cadherin/catenin complex at AJs after conversion of cadherin isoforms may imply that the mediated adhesion signals are needed for remote metastasis of cancer cell mass.

Multiple mechanisms can inactivate E-cadherin in cancer cells. Recent reports have highlighted the role of EMT regulator, snail, a strong repressor of E-cadherin gene expression in cancer cell lines^[17, 18]. Snail is a zinc finger transcription factor which directly represses E-cadherin expression by binding to E-box DNA-binding sequence CAGGTG and is required for mesoderm differentiation and neural crest formation during embryonic development. In human carcinoma and melanoma, the expression of snail correlates with the absence of E-cadherin expression^[19-21]. Forced expression of snail in cultured epithelial cells represses

the E-cadherin promoter and increases invasion and matrix metalloproteinase-2 expression^[22]. Slug, which also represses the E-cadherin promoter, can likewise induce EMT, increase cell motility and invasion and confer tumorigenesis^[23]. Twist is originally identified in *Drosophila* as a protein involved in establishing dorso-ventral polarity and in epithelial-mesenchymal transition. Recent findings indicate that twist is both an E-cadherin repressor and an EMT inducer^[24]. Furthermore, the findings implicate that twist is involved in tumor cell intravasation and in tumor metastasis. In the present study, strong expressions of snail, slug and twist were observed in the transformed gastric cell line Ges-1 and three gastric carcinoma cell lines. Meanwhile, these cell lines were all negative for E-cadherin expression but positive for N-cadherin expression.

In conclusion, EMT regulators play a critical role in human gastric carcinoma. The transcription factors (snail, slug and twist) are correlated with the alteration of cadherin isoforms in human gastric cancer cells. However, the mechanism of how the transcription factors regulate the alteration of cadherin isoforms remains to be elucidated.

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A case of an intussuscepted neuroendocrine carcinoma of the appendix

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Abstract

We have described a previously unreported entity of an intussuscepted neuroendocrine carcinoma of the appendix. Our patient was a 70-year-old man whose only complaint was insipient weight loss. Colonoscopy showed a malignant cecal "polyp", and an extended right hemicolectomy was performed. We have reviewed the literature on the causes of appendiceal intussusception and their appropriate treatment options, and clarified the classification of neuroendocrine tumors of the gastrointestinal tract.

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Key words: Neuroendocrine carcinoma; Appendix; Intussusception

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INTRODUCTION

Intussusception of the appendix is a well-recognized, though rare, entity (occurring with an incidence of 0.01% in a large autopsy series^[1]). Occasionally it is accompanied by an associated appendiceal abnormality, such as an adenoma, endometriosis, carcinoid tumor or adenocarcinoma. Typically, these cases present as acute appendicitis. Less commonly, they present with chronic episodic abdominal pain, cecal mass or even rectal bleeding, for which the patient may have to undergo an endoscopic

examination and the intussuscepted appendix may be interpreted as a cecal polyp. Occasionally, the patient has no gastrointestinal symptoms. Whereas carcinoid tumor (now termed as well-differentiated neuroendocrine tumor) is the commonest tumor of the appendix, neuroendocrine carcinoma of the appendix is a rare entity (occurring in about 0.2% of all appendectomy specimens^[2]). We here present a case of a previously unreported phenomenon of intussusception of the appendix due to a neuroendocrine carcinoma.

CASE REPORT

A 70-year-old man presented with an 18-mo history of 13 kg weight loss. He was originally referred to the rheumatologists for investigation as he had a past medical history of rheumatoid arthritis. Other past medical history included cholecystectomy and partial gastrectomy with vagotomy for perforated duodenal ulceration. He had no gastrointestinal symptoms.

He underwent an upper gastrointestinal endoscopy with no abnormalities detected. Colonoscopy revealed a lesion in the ascending colon that appeared macroscopically malignant (Figure 1). Two further benign-looking polyps were also identified, one each in the transverse colon and at the hepatic flexure. Biopsies of the ascending colon lesion suggested adenocarcinoma. A CT scan failed to demonstrate the tumor due to extensive fecal loading. There was no evidence of metastatic disease. Pre-operative blood investigations revealed a mild iron-deficiency anemia (hemoglobin 11.9 g/dL) and no other abnormality.

During the operation, a cecal tumor was found and a large lymph node was present in the mesentery. An extended right hemicolectomy was performed and an end-to-end anastomosis fashioned.

The resected specimen showed complete intussusception of the appendix, with invagination of mesoappendix fat seen at the base on the peritoneal surface. The whole of the appendix was involved by the tumor, and measured 4.5 cm in length and 2.5 cm wide at the base (Figure 2). Microscopic examination revealed a well-differentiated neuroendocrine carcinoma with a trabecular and glandular pattern (Figure 3), which was invading into the invaginated serosal fat of the appendix. Immunohistochemistry showed weak positivity with chromogranin (Figure 4) and synaptophysin, and strong membrane and cytoplasmic positivity with the low molecular weight cytokeratin (CK) marker, CAM

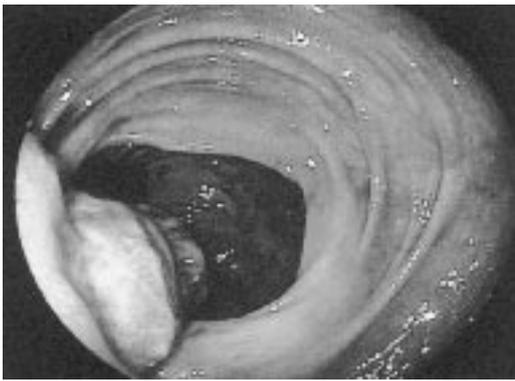


Figure 1 Endoscopic appearance of polypoid lesion in the cecum.

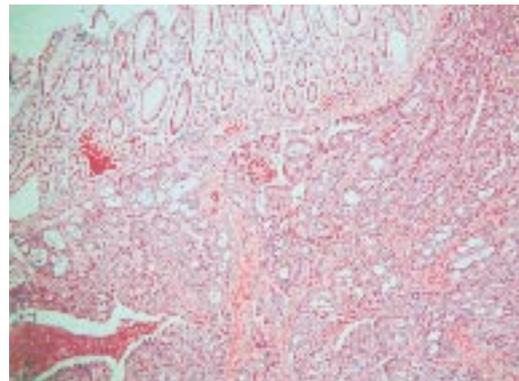


Figure 3 Well-differentiated neuroendocrine carcinoma, arranged in nests and trabeculae, beneath uninvolved large-intestinal type appendiceal mucosa (top left hand corner) (hematoxylin and eosin; $\times 16$).

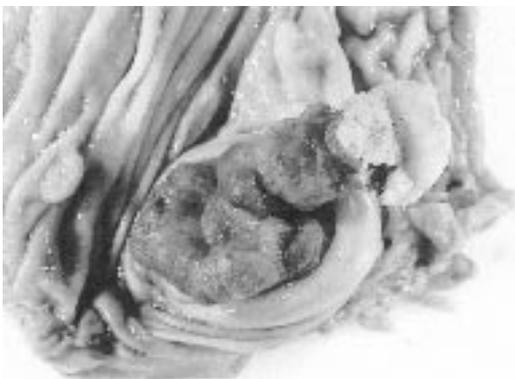


Figure 2 Gross specimen of invaginated appendiceal tumor seen on opening cecum.

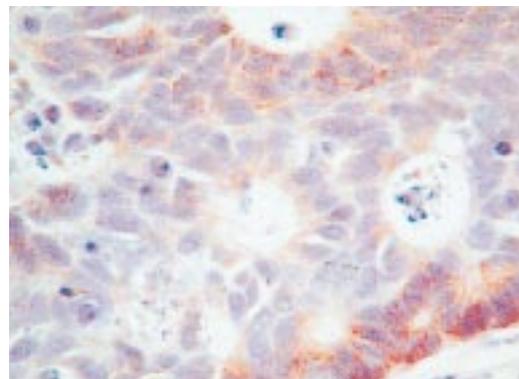


Figure 4 Chromogranin positive immunohistochemical staining (brown cytoplasmic staining) demonstrating neuroendocrine differentiation of tumor cells ($\times 40$, 1:5 000 dilution, DAK-A3 clone, DAKO, Cambridge, UK).

5.2. The tumor cells were negative for CK 20, which is usually positive in primary colorectal adenocarcinoma. The histological pattern and immunoprofile were those of neuroendocrine carcinoma. Lymphovascular invasion was identified and 1 of the 11 dissected lymph nodes contained metastatic carcinoma. The involved node was 2 cm in size and largely necrotic. Five other small polyps were also found within the specimen (2 hyperplastic, 3 adenomatous).

Post-operative recovery was uncomplicated and he was discharged 7 d post-operatively. The case was discussed at the colorectal cancer multidisciplinary (MDT) meeting and consensus was that no further adjuvant treatment was needed. He was to be followed up in the surgical clinic and was well 4 mo post-surgery.

DISCUSSION

Intussusception of the appendix is rare. In a study by Collins, of 71 000 autopsy appendiceal specimens, prevalence of intussusception was only 0.01%^[1]. Clinically it is almost never diagnosed pre-operatively, most commonly mimicking acute appendicitis. It can also present with symptoms of chronic, episodic abdominal pain with or without rectal bleeding, painless rectal bleeding, or may be asymptomatic. Cases can be diagnosed

preoperatively by means of radiological examination^[3]. At barium enema there is an absence of appendiceal filling together with a cecal-filling defect in the anticipated location of the appendix. Ultrasound scanning may show a “target-like” picture or “concentric ring sign”, though neither of these radiological appearances is specific for an intussuscepted appendix^[4]. CT scans may reveal a mass lesion within the cecum without a discernable appendix. At colonoscopy, the intussusception may be misinterpreted as a cecal polyp. Therefore, in all the cases of cecal polyp identification, the appendiceal orifice should be routinely searched for and noted to be present.

The mechanism and pathogenesis of intussusception of the appendix has been extensively studied by Fink *et al*^[5]. It may be due to anatomical causes, such as a thin mesoappendix, a mobile appendix, or a wide appendicular lumen, or due to an associated appendiceal abnormality. These include intraluminal factors (fecoliths, foreign bodies, parasites) or intramural factors (lymphoid follicle hyperplasia, endometriosis, and tumors).

Tumors of the appendix, with or without intussusception, are uncommon. Mostly they are benign entities, such as mucocoeles, adenomata, and benign carcinoids tumors. Few are malignant, occurring in only about 1% of appendicectomy specimens^[6]. In one large series, only 20% of these malignant appendiceal tumors

were neuroendocrine carcinomas^[2], the majority being adenocarcinomas. Using the WHO classification of endocrine tumors of the gastroenteropancreatic system (2000)^[7], classical benign carcinoid tumors have been incorporated under the heading of neuroendocrine tumors, and are now termed as well-differentiated neuroendocrine tumors. They are considered to be of benign or uncertain malignant potential. Those with malignant features (infiltrative growth pattern, invasion into the muscularis propria, greater than 2 cm in diameter, or with nodal metastases), previously called malignant carcinoids, are now termed as neuroendocrine carcinomas, well- or poorly-differentiated. Until now, no poorly-differentiated neuroendocrine carcinomas of the appendix have been described. Classically, well-differentiated neuroendocrine carcinomas of the appendix have organoid, nesting, trabecular, rosette-like or palisading patterns, suggestive of endocrine differentiation^[8]. They are associated with a worse prognosis than the more commonly diagnosed benign carcinoids/well-differentiated neuroendocrine tumors.

It is important that an intussuscepted appendix is managed appropriately. In those cases uninvolved by a concurrent malignant tumor, reduction at laparotomy or laparoscopy with subsequent appendicectomy, or right hemicolectomy is the surgical treatment of choice. These lesions may be misinterpreted as a broad-based cecal polyp during colonoscopy, and subsequent endoscopic resection may result in unexpected complications, such as perforation and peritonitis^[9]. Therefore, wide-stalked cecal polyps, where the appendiceal orifice cannot be visualized, should be approached with caution endoscopically. However, in the circumstances when a malignant tumor of the appendix is also present, right

hemicolectomy or ileocecal resection with lymph node dissection should be performed^[1]. Though a diagnosis of appendiceal intussusception had not been pre-operatively made in our patient, he underwent the correct treatment of an extended right hemicolectomy, because of the pre-operative diagnosis of a presumed malignant cecal polyp.

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CASE REPORT

Subfulminant hepatitis B after infliximab in Crohn's disease: Need for HBV-screening?

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Abstract

Infections are a major adverse effect during the treatment with anti-TNF- α . While exclusion of any bacterial infection and screening for tuberculosis are mandatory before initiating a therapy with anti-TNF- α -antibodies, there are no guidelines whether to screen for or how to deal with chronic viral infections such as hepatitis B. In this case report, we have described a patient with Crohn's disease who developed subfulminant hepatitis B after the fourth infusion of infliximab due to an unrecognized HBs-antigen carrier state. He recovered completely after lamivudine therapy was started, but this severe adverse event could have been prevented if screening for HBV and pre-emptive therapy with lamivudine would have been started prior to infliximab. We therefore strongly argue in favor of extended screening recommendations for infectious diseases including viral infections before considering a therapy with infliximab.

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Key words: Hepatitis B; TNF alpha; Lamivudine

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INTRODUCTION

Infliximab has become the standard therapy for refractory,

steroid-dependent or fistulizing Crohn's disease^[4,14,19]. The association between the use of this monoclonal chimeric mouse antibody, and increased risk of severe bacterial infections^[16] and reactivation of tuberculosis^[6] has received much attention. The influence of infliximab on viral infections, however, has not been extensively investigated, and data that are available on reactivation of chronic viral infections such as hepatitis B and C and EBV are conflicting. Reijasse *et al*^[15] studied the viral kinetics of EBV in patients receiving immunosuppressive drugs including infliximab and found no increased viral load in them compared to the control group. Several case studies and investigations of small patient cohorts have reported that Crohn's disease in patients with documented pre-existing chronic hepatitis B or C was successfully treated with infliximab, without the drug causing any deterioration of the liver disease^[11,13,21]. In contrast to these publications, in their prospective study of Crohn's patients with coexisting chronic HBV-infection treated with infliximab, Esteve *et al*^[1] observed two severe hepatitis flare-ups during treatment with this drug. Ostuni *et al*^[12] reported on a patient with reactivated hepatitis B after therapy with infliximab and methotrexate. Michel *et al*^[8] observed fulminant hepatic failure in a patient with inactive chronic hepatitis B after being treated with infliximab but without signs of HBV-reactivation and therefore of unknown etiology.

Several authors have recommended pre-emptive treatment with lamivudine of HBV-carriers with Crohn's disease before starting infliximab-therapy^[1,8].

FDA-recommendations released in December 2004 also alert physicians to the danger of primary hepatotoxicity or reactivation of chronic viral hepatitis caused by the administration of infliximab.

CASE REPORT

We report here on a 50-year-old patient in whom Crohn's disease with terminal ileitis was diagnosed in January 2001. Initially he received a course of mesalamine. This, proving to be ineffective, was followed by a course of systemic steroids together with mesalamine. Steroid withdrawal was difficult, but in June 2003 steroids were discontinued and the patient was in complete remission until February 2004 when he had a relapse with abdominal pain and loose and bloody stools. Treatment was restarted with budesonide 3 mg t.i.d. and azathioprine 150 mg. Budesonide failed to improve the symptoms and steroid-therapy was switched

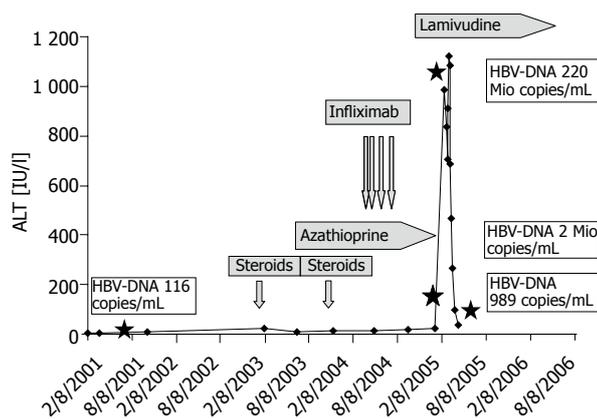


Figure 1 ALT remained within the normal range during therapy with prednisolone, azathioprine and during the infusions with infliximab. Only after the fourth infusion of infliximab, the patient developed a fulminant hepatitis with ALT up to 1 100 IU/L. HBV-DNA () quantification showed 220 mio copies/mL. Lamivudine (150 mg/d) was started and led to a rapid decrease in transaminases and HBV-DNA.

to systemic steroids (methylprednisolone 40 mg) again. Abdominal pain and bloody stools improved under this regimen but tapering of steroids was followed by an immediate relapse despite azathioprine 2 mg/kg for several months. Endoscopy showed ulcerating inflammation in the terminal ileum and capsule endoscopy revealed involvement of the distal jejunum. Bowel surgery was discussed with the patient, but he wished to try every possible medical treatment before going for surgery. We therefore re-assessed the situation and decided to add infliximab 5 mg/kg (total amount 400 mg) because of steroid-dependent disease. Tuberculosis was excluded by tuberculin-testing and chest X-ray. Blood tests showed mild leukocytosis (14 g/L), all other results including renal and liver function tests, c-reactive protein, iron metabolism, and vitamins were within normal ranges. There was no history of any other disease before the diagnosis of Crohn's disease, besides a mild reactive depression for which the patient has been on mirtazapine for more than a year. Transaminases were documented to be within normal ranges since 2001.

Infliximab was administered successfully at week 0, 2, and 6 followed by complete remission and rapid tapering of steroids. Basis therapy consisted at this time of azathioprine 150 mg, mirtazapine 30 mg, pantoprazole 40 mg and brotizolam 0.25 mg at night. The infusion at wk 6 was followed by 3 d of flu-like symptoms. One month after the third infliximab infusion, the patient came to the outpatient clinic because of abdominal discomfort and malaise. Blood tests showed signs of acute hepatitis (ALT 983 [normal <50 IU/L], AST 413 [normal <50 IU/L], γ GT 109 [normal <66 IU/L], LDH 237 [normal <232 IU/L], bilirubin 2.17 [normal <1.28 mg/dL] and a decreased prothrombin time of 63% [normal >70%]) (course of ALT Figure 1). Liver parenchyma was hyperechogenic in sonography but there were no signs of liver cirrhosis and as expected, there were no signs of mechanical cholestasis. The patient mentioned at this point that he had been immunized against hepatitis A and B six months before by his general practitioner because all other members of his family had undergone hepatitis B and he

intended to travel to Southern Europe during the summer holidays. No previous serological examination had been performed before immunization.

Complete work-up was done one week after his admission to the hospital. Hepatitis-serology revealed immunity against hepatitis A and negative serology and PCR for hepatitis C, but circulating HBs-antigen, no HBe-antigen but anti-HBe- and anti-HBc-antibodies, and negative anti-HBc-IgM were detected. Quantification of HBV-DNA showed >220 000 000 copies/mL (38 000 000 IU/mL). Other causes for hepatitis such as EBV, CMV, autoimmune hepatitis and acute Wilson's disease were ruled out. We suspected reactivation of hepatitis B from a previously unknown HBV-carrier state. This was corroborated by serology from a frozen serum sample from a previous visit in 2001 also showing the features of an HBs-antigen carrier but a low viral load with 116 copies/mL (20 IU/mL).

Antiviral therapy (lamivudine 150 mg daily) was started immediately after diagnosis. Despite the therapy, liver synthesis parameters transiently decreased and reached a nadir three weeks after the first manifestation with a prothrombin-time of 42% and bilirubin peak of 35 mg/dL. At the same time, the patient developed mild hepatic encephalopathy with increased sleepiness. One month after the diagnosis, the patient recovered, transaminases decreased and prothrombin-time normalized and HBV-DNA dropped to 2 000 000 copies/mL (340 000 IU/mL). The patient could be discharged and followed up in the outpatient clinic. The most recent visit two months after the acute hepatitis showed normal transaminases and HBV-DNA of 989 copies/mL (170 IU/mL).

DISCUSSION

While patients undergoing chemotherapy for malignancy have long been screened for chronic hepatitis B and treated prophylactically with lamivudine if positive, this has not been performed on a routine basis in patients with chronic inflammatory bowel disease requiring immunosuppressive regimens including infliximab. Our knowledge about the role of TNF- α in chronic viral hepatitis is sparse but there is evidence that TNF- α synergizes with interferons in suppressing viral replication^[2,7,22] and is essential in clearing HBV^[17]. A promoting effect for increased HBV-replication during treatment with anti-TNF-antibodies could be explained on this basis. But why HBs-antigen carriers, upon anti-TNF- α treatment, suddenly lose their tolerance against HBV and switch to a cytotoxic T-lymphocytic activation leading to hepatocyte injury and liver failure remains to be elucidated. At present we can only speculate that TNF- α might play an important role in maintaining tolerance in the HBs-antigen carrier state.

Till date there are only three case reports and one report of a small group of HBs-antigen carriers that suffered HBV-reactivation following administration of infliximab. Four patients developed fulminant hepatitis B^[1,8,12] while one patient had only moderately elevated transaminases^[20] that returned to normal without antiviral therapy. Esteve *et al* reported that two of their patients

showed severe reactivation and one died from hepatic failure, but another patient on lamivudine had no worsening of liver disease during infliximab-therapy^[1]. Stable HBV under lamivudine has also been reported by Oniankitan *et al*^[11] and Valle Garcia-Sanchez *et al*^[21]. Keeping these reports in mind and looking for other factors that might influence the course of HBV, we suggest that (1) the number of infliximab-infusions and (2) concomitant immunomodulatory therapy might influence the course of HBV-reactivation (azathioprine in our case and methotrexate therapy in the other cases with severe hepatitis).

In our patient HBV-reactivation was noted after the third infusion of infliximab. Previous treatment with steroids and azathioprine alone had not induced a flare-up in hepatitis B, although it is known that all types of immunosuppressive therapy can lead to a flare-up in hepatitis B. For steroids this has been documented most frequently in patients with hematological malignancies^[18]. Azathioprine and methotrexate have also been linked with HBV-recurrence. But while this occurred on treatment with azathioprine^[9], it took place only after the discontinuation of methotrexate^[3,5,10].

In conclusion, our report on a patient with Crohn's disease developing subfulminant hepatitis under infliximab treatment due to reactivation of a previously unknown HBs-antigen-carrier state draws attention to the need for screening patients with inflammatory bowel disease for hepatitis B and for antiviral prophylaxis in HBV-positive patients prior to therapy with anti-TNF- α antibodies.

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A case of Crohn's disease involving the gallbladder

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Abstract

Crohn's disease is well known to affect any part of the gastrointestinal tract including the oral cavity and anus. Various extraintestinal complications have been reported in Crohn's disease, but extraintestinal involvement characterized by granulomatous lesions is uncommon. Here, we have reported a case about the involvement of the gallbladder in Crohn's disease. A 33-year-old woman was diagnosed having panperitonitis due to intestinal perforation and cholecystitis. The patient was moved to the surgical service for an emergency operation. On the resected specimen, there was a broad longitudinal ulcer at the mesenteric side. The mucosa of the gallbladder was nodular and granular, and the wall was thickened. The surface epithelium of the gallbladder was partially eroded and pyloric gland metaplasia was observed focally. Rokitansky-Aschoff sinuses were also present. From the lamina propria to the subserosal layer, there were several well-formed epithelioid cell granulomas, which were the non-caseating sarcooidal type different from the foreign-body and xanthomatous granulomas. Periodic-acid Schiff and acid fast stains revealed no organism within the granulomas. Lymphoid aggregates were present throughout the gallbladder wall. Sections from the resected ileum showed typical features of the Crohn's disease. When cholecystectomy is performed in a patient with Crohn's disease, the possibility of gallbladder involvement should be carefully examined by histopathological tests.

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Key words: IBD; Granuloma; Inflammation

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INTRODUCTION

Crohn's disease is well known to affect any part of the gastrointestinal tract including the oral cavity and anus. Various extraintestinal complications have been reported in Crohn's disease, but extraintestinal involvement characterized by granulomatous lesions is uncommon. Here, we report a case of the involvement of the gallbladder in Crohn's disease.

CASE REPORT

The patient was a 33-year-old woman who was admitted to the Shiga University of Medical Science Hospital with complaints of nausea, vomiting, and severe abdominal pain, which had lasted for a couple of hours. Her past history included Crohn's disease (ileocolonic type) and gallbladder stone, which had been diagnosed 2 years ago. She was well controlled with medications (2 250 mg/d 5-aminosalicylate and 2 000 kcal/d elemental diet) in these 2 years. She did not feel any symptoms of gallbladder stone.

At the time of admission, her vital signs were as follows: body temperature 37.4 °C, blood pressure 92/60 Pa, and pulse 98 beats/min. The laboratory data included a white blood count of 13.1 ($\times 10^3$ /mL), and C-reactive protein of 13.6 mg/dL. The values for biochemistry were within the normal range. An abdominal CT scan demonstrated a free-air space located in the dorsal side of the abdomen, ascites, strong contrast enhancement of a thickened ileum, fluid collection in the small intestine, and gallbladder swelling with calcifications (Figure 1). The intra- and extrahepatic bile ducts appeared normal. These findings were suggestive of panperitonitis due to intestinal (ileal) perforation and cholecystitis.

After the admission, the patient was moved to the surgical service for an emergency operation. A perforating hole (diameter 5 mm) was located at the ileum 20 cm oral side from Bauhin's valve, and thus segmental ileotomy (25 cm resection) was performed. On the resected specimen, there was a broad longitudinal ulcer at the mesenteric side. Following an ileotomy, the patient also received a cholecystectomy. The serosal surface of the gallbladder adhered tightly to the greater omentum and duodenal bulb.

The mucosa was nodular and granular, and the wall was

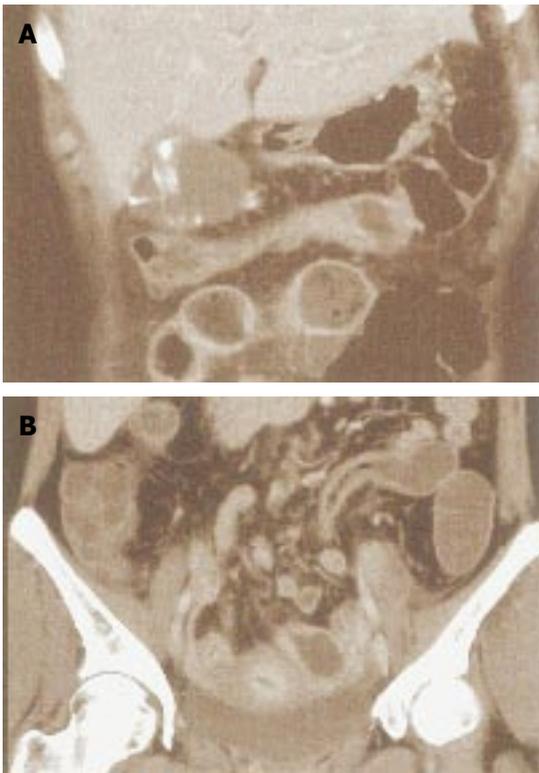


Figure 1 Abdominal CT scan on admission. CT scan demonstrated gallbladder swelling with thickened wall and stones, ascites, a thickened ileum, and fluid collection in the small intestine.

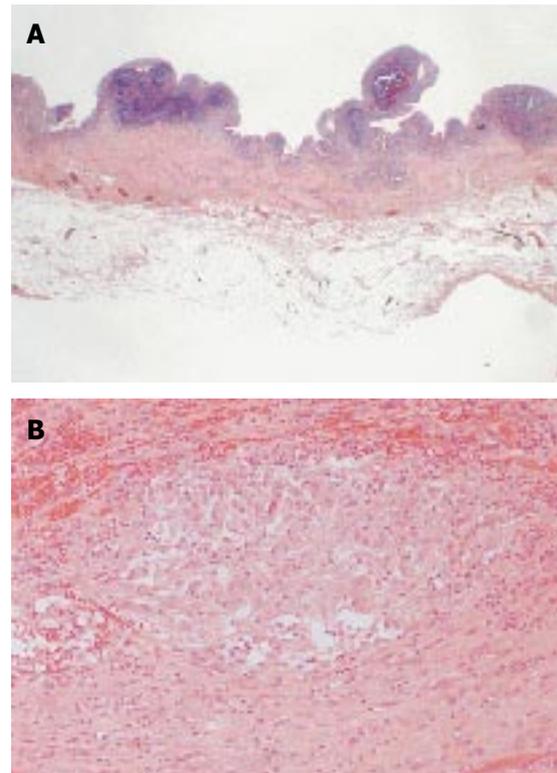


Figure 2 Histological findings of the gallbladder. **A:** The mucosa was nodular and granular, and the wall was thickened. Erosion and submucosal bleeding were also observed, but there were no obvious ulcers; **B:** There were several well-formed epithelioid cell granulomas, which was non-caseating sarcoid-like type, different from the foreign-body and xanthomatous granulomas.

thickened. Erosion and submucosal bleeding were also observed, but there were no obvious ulcers (Figure 2A). The surface epithelium of the gallbladder was partially eroded and pyloric gland metaplasia was observed focally. The lamina propria showed discontinuous chronic active inflammation. The muscular layer was hypertrophied and the subserosal layer was thickened with marked fibrosis. Rokitansky-Aschoff sinuses were also present. From the lamina propria to the subserosal layer, there were several well-formed epithelioid cell granulomas, which were the non-caseating sarcoid-like type, different from the foreign-body and xanthomatous granulomas (Figure 2B). Periodic-acid Schiff and acid fast stains revealed no organism within the granulomas. Lymphoid aggregates were present throughout the gallbladder wall. Sections from the resected ileum showed typical features of Crohn's disease.

Her clinical course was good, and she was discharged from the hospital 1 mo after the operation.

DISCUSSION

A high prevalence of gallstones in Crohn's disease has been reported^[1], but gallbladder involvement in Crohn's disease is very rare. Based on our MEDLINE search of the literature, only three case reports were found^[2-4]. In all

cases including our patient, the pathological examination of the gallbladder revealed transmural inflammation, granulomatous change and lymphoid aggregation, all of which were similar to those seen in the bowel lesions. Duodenal Crohn's disease has been reported to occasionally involve the surrounding organs^[2,3], but our case did not present any duodenal lesions, indicating that the gallbladder lesion might not be a consequence of spread by contiguity. When cholecystectomy is performed in a patient with Crohn's disease, the possibility of gallbladder involvement should be carefully examined by histopathological examinations.

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Intestinal Behcet's disease with pyoderma gangrenosum: A case report

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Abstract

We report here a very rare case of intestinal Behcet's disease with pyoderma gangrenosum. A 16-year-old woman who was diagnosed with intestinal Behcet's disease by the presence of cutaneous pathergy together with two major criteria (oral and genital aphthoses) and one minor criterion (gastrointestinal manifestations), was referred to our hospital with a left lower leg ulcer and abdominal pain in September 1989. Colonoscopy demonstrated flare-up colitis involving the entire colon. Her lower leg lesion was a painful destructive ulcer with an irregular margin and a ragged overhanging edge. Based on these clinical and laboratory findings, we diagnosed her cutaneous ulcer as pyoderma gangrenosum developing with exacerbated intestinal Behcet's disease. Her cutaneous and intestinal lesions were poorly controlled though she received oral prednisolone treatment for a month. Because of aggravated abdominal symptoms with peritoneal irritation, we performed total colectomy in November 1989. The resected specimen was histologically compatible with intestinal Behcet's disease showing severe inflammation with deep ulcerations and neutrophil accumulation. Subsequently, pyoderma gangrenosum rapidly improved. This clinical course may suggest the close relationship between pyoderma gangrenosum and intestinal Behcet's disease.

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Key words: Behcet's disease; Pyoderma gangrenosum

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INTRODUCTION

Behcet's disease is a multi-system inflammatory disorder of unknown origin, characterized by recurrent oral and genital ulcerations, ocular and cutaneous lesions, arthritis and vascular disease. Cases of Behcet's disease cluster along the ancient Silk Road, which extends from eastern Asia to the Mediterranean basin^[1-3]. It is often difficult to distinguish Behcet's disease from other inflammatory bowel diseases. Pyoderma gangrenosum is an idiopathic ulcerative skin disease. Because the histological findings of pyoderma gangrenosum are nonspecific and no laboratory confirmation exists, the diagnosis is based primarily on its clinical manifestations. Though this skin lesion is known to be associated with inflammatory bowel diseases, such as ulcerative colitis and Crohn's disease, pyoderma gangrenosum is very rare in Behcet's disease patients. We present a patient with extensive pyoderma gangrenosum whose skin lesions were resolved after surgical removal of the inflamed intestinal lesions.

CASE REPORT

A 16-year-old woman was referred to our hospital in September 1989 due to painful ulceration on the left foot and lower abdominal pain. She was diagnosed with intestinal Behcet's disease by the presence of cutaneous pathergy together with two major criteria (oral and genital aphthoses) and one minor criterion (gastrointestinal manifestations) in 1984. Her lower leg lesion was a painful destructive ulcer with an irregular margin and a ragged overhanging edge (Figure 1A). She was negative for repeated bacterial, fungal and viral cultures. Colonoscopy demonstrated flare-up colitis involving the entire colon. Based on these clinical and laboratory findings, her skin lesion was considered pyoderma gangrenosum developing with aggravation of her intestinal Behcet's disease. She was treated with prednisolone (20 mg orally per day) for a month, but the skin lesion was poorly controlled. In November 1989, she complained of severe abdominal pain and melena. Laboratory data showed a white blood cell count of 14900/mm³, serum C-reactive protein of 4.5 mg/dL and hemoglobin of 12.3 g/dL. Because her abdominal symptoms were aggravated with peritoneal irritation, we performed a total colectomy with partial ileolectomy and ileo-rectal anastomosis. The resected specimen revealed severe inflammation with punched-out ulcerations of the colon and ileum (Figure 2A). Microscopic findings showed active colitis with neutrophil

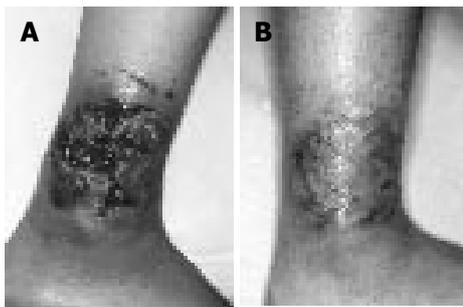


Figure 1 Painful destructive ulcer of the foot with an irregular margin and a ragged overhanging edge (A) and intractable leg ulcer without improvement after oral corticosteroid therapy was cured after total colectomy (B).

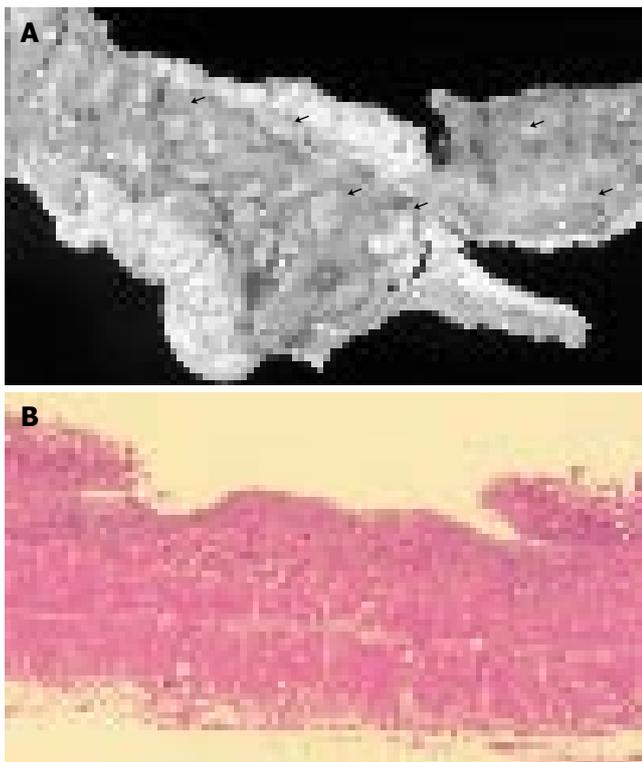


Figure 2 Severe inflammation and deep ulcerations of specimens from colon and ileum (A) and active colitis with neutrophil accumulation around the ulcer beds (B) (HE stain, x100).

accumulation around the ulcer beds. Subsequently her abdominal symptoms gradually improved. Surprisingly, intractable ulceration of the left foot disappeared without scarring within two weeks after the surgery (Figure 1B). She was discharged with an uneventful postoperative course. Relapse of pyoderma gangrenosum has not been observed for 10 years after surgery.

DISCUSSION

Intestinal Behcet's disease has been categorized in a family with inflammatory bowel disease, and causes abdominal pain, diarrhea, melena and even perforation. The ileocecal region is the most commonly affected part of the gastrointestinal tract, but the distal colon may also be involved. It is often difficult to distinguish Behcet's disease

Table 1 Reported cases of Behcet's disease with pyoderma gangrenosum

| Author | Year | Age | Gender | Preceding disease | Treatment |
|-------------------------------------|------|-----|--------|-------------------|-----------------|
| Munro <i>et al</i> ^[5] | 1988 | 43 | Female | Behcet | Thalidomide |
| Lilford <i>et al</i> ^[6] | 1989 | 40 | Female | Behcet | Steroid |
| Rustin <i>et al</i> ^[7] | 1990 | 40 | Male | Behcet | Thalidomide |
| Armas <i>et al</i> ^[8] | 1992 | 52 | Male | Behcet | Steroid |
| Present case | 2003 | 16 | Female | Behcet | Total colectomy |

from the other inflammatory bowel diseases because of the similar extra-intestinal manifestations including oral aphthosis, uveitis and arthritis. Genital ulceration and positive pathergy test are the extra-intestinal features of Behcet's disease alone, which are not seen in the other inflammatory bowel diseases. Our patient was diagnosed as Behcet's disease by the presence of cutaneous pathergy together with two major criteria (recurrent oral and genital aphthoses) and one minor criterion (gastrointestinal manifestations)^[4].

In Behcet's disease, multiple cutaneous lesions, such as erythema nodosum, folliculitis and pustules are seen, but pyoderma gangrenosum is very rare. Pyoderma gangrenosum in the present case did not respond to oral corticosteroids but was improved soon after the resection of inflamed intestinal lesions. Since the first report by Munro and Cox in 1988^[5], four cases of pyoderma gangrenosum associated with Behcet's disease have been reported in English literature^[6-8] (Table 1). Behcet's disease always precedes the onset of pyoderma gangrenosum. These patients are treated with corticosteroids, immunosuppressants or thalidomide. It was reported that cases of pyoderma gangrenosum had no improvement after surgical removal of the intestinal lesions^[9]. Pyoderma gangrenosum usually responds to systemic corticosteroids and may also be rapidly improved by surgical resection of the associated diseases, such as ulcerative colitis and Crohn's disease. Therefore, the activity of pyoderma gangrenosum is closely related to the activity of associated diseases. In the present case, pyoderma gangrenosum rapidly improved after total colectomy. The skin manifestations of Behcet's disease are classified as neutrophilic vascular reaction^[10]. The pathergy test is useful for evaluating skin irritability and is a diagnostic criterion of Behcet's disease. However, this test can also be positive in other diseases such as Sweet's syndrome and pyoderma gangrenosum. Pyoderma gangrenosum is a form of cutaneous vasculitis^[11] and accumulation of neutrophils and mononuclear cells is observed at the reaction site. In both Behcet's disease and pyoderma gangrenosum, primary pustular lesions can occur and neither of them has a characteristic histological appearance. They may belong to the category of the neutrophilic dermatoses.

Though the relationship between Behcet's disease and pyoderma gangrenosum is not referred in diagnostic criteria of Behcet's disease, the occurrence of pyoderma gangrenosum may be highly relevant with the pathogenesis of Behcet's disease.

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CASE REPORT

Spontaneous rupture of a type IV_A choledochal cyst in a young adult during radiological imaging

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CASE REPORT

A 24-year-old male was admitted to our hospital with jaundice and epigastric pain reflecting low in the back. The body temperature was normal. He was a heavy drinker in the previous 4 years and reported a history of infantile hyperbilirubinemia, which resolved spontaneously. He had positive Murphy and Giordano signs and a palpable epigastric mass. The laboratory examination results above normal range were as follows: WBC: 12 500/ μ L (neutrophil: 93.6%, lymphocyte: 5.1%), SGOT: 159 IU/L, SGPT: 559 IU/L, γ GT: 520 IU/L, ALP: 244 IU/L, LDH: 699 IU/L, CPK: 606 IU/L, bilirubin (total): 7.68 mg/dL, bilirubin (direct): 6.5 mg/dL, and bile in the urine.

Computerized tomography (CT) of the upper abdomen was ordered, and displayed a 9 cm \times 10 cm cyst in the common bile duct (CBD) region, extending from porta hepatis down to the level of the third portion of the duodenum (Figure 1). Distension of intrahepatic bile ducts with stasis was also observed. The findings were consistent with a choledochal cyst (CC). Further investigation with magnetic resonance imaging (MRI) and MR cholangiopancreatography (MRCP) was considered appropriate. It confirmed the intrahepatic duct distension and the large cyst (Figure 2). It was considered to be a large CC type IV_A. During MRI, the patient developed "acute abdomen" symptoms. Spin echo T₂-weighed images and MRCP depicted bile leak in the peritoneal cavity (Figure 3).

Immediately after MRCP he underwent urgent abdominal surgery. A 1-cm rupture of the distended CBD was found, with a considerable amount of intraperitoneal bile and focal biliary peritonitis. A Kehr tube was inserted into the CBD at the site of the rupture and the lips of the lesion were appropriately stitched.

In order to verify the adequacy of the urgent surgical intervention, ^{99m}Tc-mebrofenin hepatobiliary scintigraphy was advocated on the second postoperative day. The study included a 30-min dynamic acquisition, beginning with injection and consisting of 60 30-s images (frames) of the abdomen, followed by planar static images of the upper abdomen at 1½ h and 24 h post-injection. No active leaking in the peritoneal cavity was observed throughout the study. A well-circumscribed fusiform intense tracer

Abstract

A case of a 24-year-old male with jaundice and epigastric pain is reported. The patient underwent a thorough clinical, laboratory, and imaging investigation. Computerized tomography revealed a 9 cm \times 10 cm choledochal cyst. Magnetic resonance imaging and magnetic cholangiopancreatography were performed, during which he developed an "acute abdomen", with radiological evidence of biliary peritoneal leak. Urgent surgery revealed rupture of the distended malformed common bile duct. A peritoneal drain was instilled and a more definitive surgical procedure was accordingly scheduled. Hepatobiliary scintigraphy following surgery verified these findings, as well as confirmed the adequacy of the urgent surgery. A combination of radiological and nuclear medicine techniques substantially contributes to the diagnosis of choledochal cyst rupture and the adequacy of surgical intervention.

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Key words: Choledochal cyst; Rupture; MRCP; Hepatobiliary scintigraphy

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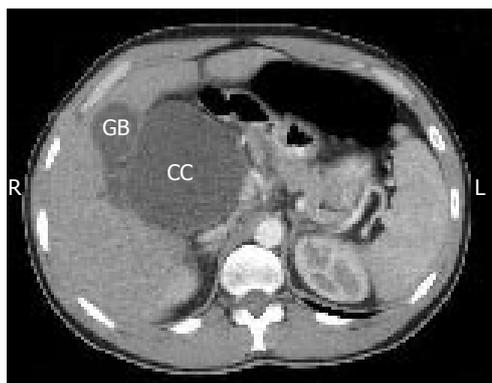


Figure 1 Upper abdominal CT (after intravenous administration of contrast material) demonstrating the extrahepatic portion of the choledochal cyst (CC). GB: gallbladder.

Table 1 Classification of choledochal cysts (after Todani *et al.*^[31])

| Type | Characteristics |
|-----------------|---|
| I _A | Dilatation of the common bile duct (CBD), with marked dilatation of part or the entire extrahepatic biliary tree and normal intrahepatic biliary tree |
| I _B | Focal and segmental dilatation of the distal CBD |
| I _C | Fusiform dilatation of the CBD, with diffuse cylindrical dilatation of the common hepatic duct and CBD as well as normal intrahepatic biliary tree |
| II | Diverticulum of the CBD |
| III | Choledochocele of the intraduodenal portion of the CBD |
| IV _A | Multiple cysts involving both the intrahepatic and extrahepatic bile ducts |
| IV _B | |
| V | Multiple cysts of the extrahepatic ducts only |
| | Caroli's disease |

accumulation was observed in the right hepatic lobe near the hepatic hilum. It appeared early in the dynamic phase (Figure 4) and was most prominent in the 90-min static image while not apparent in the 24-h image (Figure 5). It corresponded to the intrahepatic moiety of the CC. A much fainter bulbous accumulation was displayed in the left hepatic lobe, extending well beyond the liver margins, and corresponded to the large extrahepatic CC moiety. It was apparent earlier in the dynamic study and still visible in the 24-h image (Figures 4 and 5).

DISCUSSION

Choledochal cysts are congenital abnormalities of the bile ducts of unknown etiology. They comprise cystic dilations of the extrahepatic and/or intrahepatic biliary tree^[1]. They are typically described as a childhood disease. Sixty percent of the patients are diagnosed during the first decade of life. Congenital cysts occur in between 1/100 000 and 1/2 000 000 live births and are more

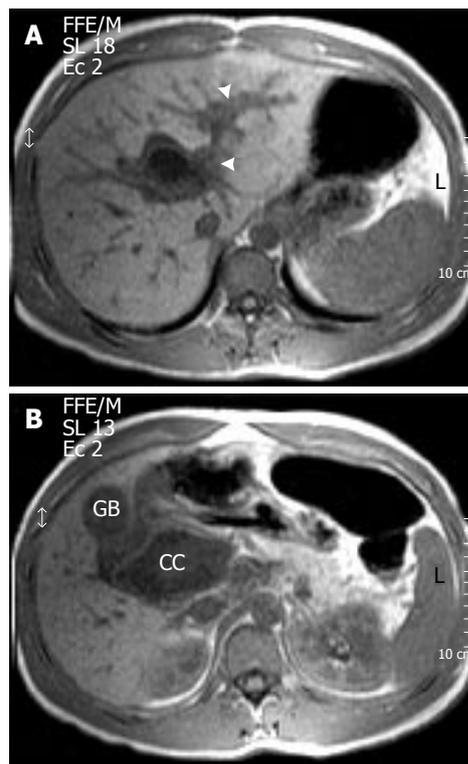


Figure 2 Significant distension of the intrahepatic bile ducts (arrowhead) in MRI of the upper abdomen (gradient echo T₁-weighted acquisition) (A). The walls of the choledochal cyst (CC) tend to acquire a concave shape, while its volume appears reduced as compared to the CT findings, both signs are considered as indicative of rupture (B). GB: gallbladder.

frequent in females (female-to-male ratio: 3:1 to 4:1). They are usually observed in Asian countries, especially in Japan.

Alonso-Lej *et al.*^[2] first classified CC into three major types: cystic, diverticular, and choledochocele. Todani *et al.*^[31] modified the classification system, which currently includes five types based on cholangiographic morphology and number of intrahepatic and extrahepatic bile duct cysts (Table 1). With regard to the shape of extrahepatic and intrahepatic ducts, type IV is further classified into three subtypes: cystic-cystic, cystic-fusiform, and fusiform-fusiform. Cystic-fusiform IV_A is the subtype that corresponds to our findings. In this particular subtype, intrahepatic dilatation occurs in both hepatic lobes, with the left being predominantly involved. Yet, in the present case it was the right lobe that was affected.

Ultrasonography is the initial screening examination of choice in suspected CC. Depending on the radiologist's skill it may reveal the type of CC. Nevertheless, inherent limitations (e.g. gas in the bowel, intra-abdominal inflammatory processes, *etc.*) allow for limited anatomic delineation of the non-dilated biliary system^[1].

CT imaging can be relied on with a high degree of confidence in confirming an unambiguous diagnosis and providing information regarding CC relationship to the surrounding structures. It usually demonstrates a cyst, which is readily distinguished from the gallbladder as in our case. Depending on patient's age and history, the cystic wall is thickened, especially on the ground of recurrent inflammation and cholangitis.

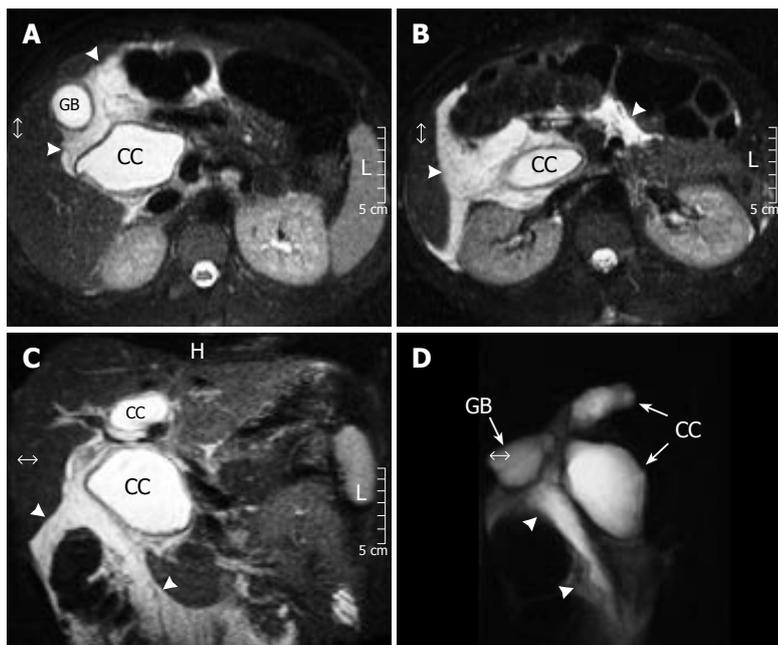


Figure 3 Abdominal MRI (spin echo T₂-weighed, transverse projections - **A** and **B**, coronal projection - **C**) displaying bile leakage into the peritoneal cavity (arrowheads). The study was followed by MRCP (turbo spin echo T₂-weighed acquisition - **D**) which imaged the bile leak (arrowheads) and moreover revealed the "egg-timer" bicameral shape of the extrahepatic moiety of the choledochal cyst (CC). GB: gallbladder.

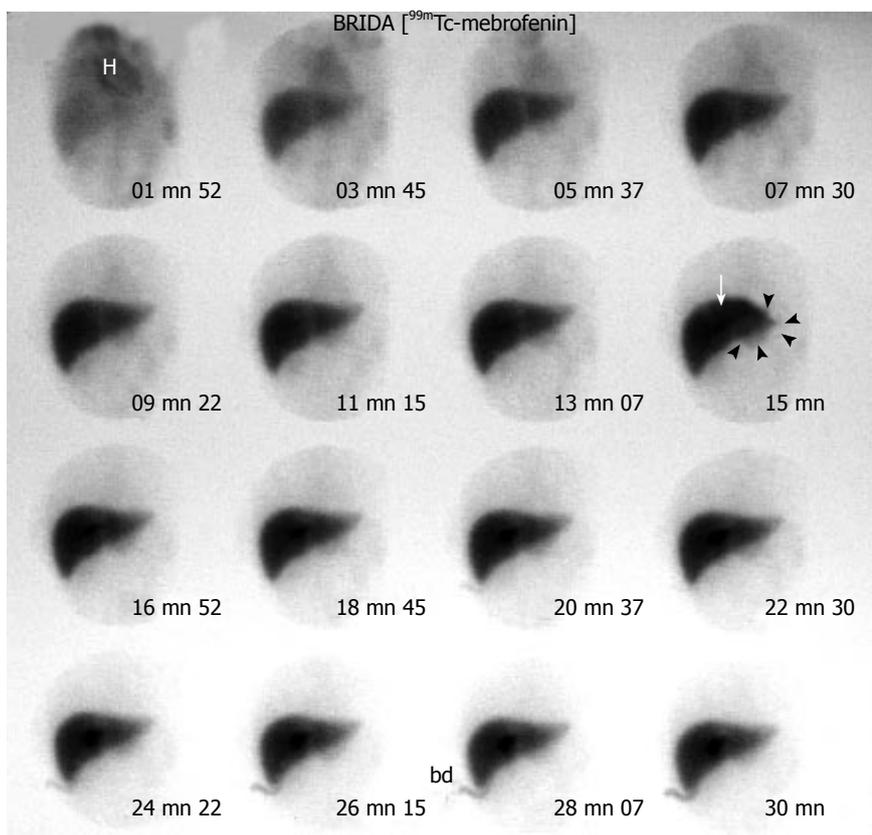


Figure 4 Postoperative ^{99m}Tc-mebrofenin dynamic study. A well-circumscribed fusiform tracer accumulation in the right hepatic lobe near the hepatic hilum is clearly visible from the 15th min of the study (arrow), and corresponds to a dilated intrahepatic duct. A much fainter bulbous accumulation is apparent in the area of the left hepatic lobe, extending well beyond the liver margins (arrowheads) and corresponding to the extrahepatic portion of the choledochal cyst. H: heart blood pool; bd: biliary drainage.

MRI with MRCP is the noninvasive imaging modality of choice for the diagnosis of CC. The cysts appear as large fusiform or saccular dilatations producing a specific signal on T₂-weighed images. Associated bile duct anomalies can also be demonstrated. On the basis of reported results, MRCP has an estimated diagnostic accuracy of 82-100%^[1]. Overall, MRCP is a promising technique that may offer as much as endoscopic retrograde cholangiopancreatography (ERCP).

Hepatobiliary scintigraphy is used in the setting of

suspected acute cholecystitis, peritoneal biliary leak, and for the investigation of neonatal jaundice. In addition, it contributes to the diagnosis of CC, although such studies are practically acquired in a limited number of patients. Rajnish *et al*^[4] evaluated the efficacy of hepatobiliary scintigraphy in classifying CC patients with confirmed Todani types I and IV cysts and found that it has a good accuracy in the diagnosis, correlating with ERCP or surgical findings in 86% of the cases. The false negative results are mainly attributable to its limitation in identifying

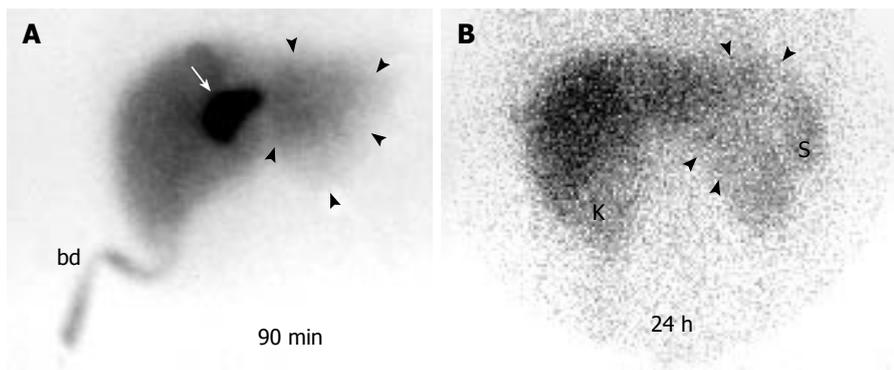


Figure 5 Planar static scintigraphic images of the upper abdomen. At 90-min post-injection the faint tracer accumulation corresponding to the extrahepatic portion of the choledochal cyst (CC) extends beyond the liver margins (arrowheads), while a fusiform tracer accumulation in a cystic dilated intrahepatic duct is prominent (arrow) (A). The latter finding is not visible in the late (24 h) image, nor bowel or peritoneal activity (B). bd: biliary drainage; K: kidney; S: spleen.

small intrahepatic dilatations in type IV_A. Nevertheless, in our case the fusiform tracer accumulation in the right hepatic lobe corresponds to a dilated intrahepatic duct forming a cyst and its location is uncommon (cysts are usually seen in the left lobe). This intrahepatic cyst was not a continuous extension of the extrahepatic moiety of the CC, but developed secondary to a hepatic hilum ductal stricture, a feature distinguishing CC type IV_A from type I^[3]. Furthermore, hepatobiliary scintigraphy can provide useful information regarding liver function, biliary patency, and biliary leak in the peritoneum.

Type IV_A is observed in 30-40% of CC cases^[5]. It is often accompanied with a primary ductal stenosis around the hepatic hilum, which causes either cystic or fusiform dilatation of the intrahepatic duct. The fusiform dilatation imaged in the present case mainly resulted from stricture, whereas cystic dilatation may develop due to primary stricture combined with the weakness of the intrahepatic duct epithelium. The stricture can be radiologically detected and needs cautious surgical exploration^[3]. The primary management of type IV_A cysts is still controversial. The intrahepatic dilatation tends to gradually reduce in size following sufficient biliary drainage. In addition, lateral hepatic segmentectomy may be necessary, especially in cases of strictures of the upper intrahepatic ducts and when the left hepatic lobe contains gallstones^[6]. Patients diagnosed with type IV CC are usually adults, who commonly develop hepatolithiasis^[7]. Formation of gallstones leads to the dilatation of the ducts and sometimes is complicated by intrahepatic abscess^[8].

Surgical treatment should be recommended to reduce the risk of other serious complications, such as cholangitis, pancreatitis, rupture, portal hypertension, cirrhosis, and cholangiocarcinoma. Pancreatitis develops in 30-70% of adults with CC^[9]. Cholangiocarcinoma may evolve in all kinds of cysts, but types I and IV are associated with a higher incidence. In these patients the prognosis is the same as in other cases of cholangiocarcinoma in the general population^[10].

On rare occasions, adults may present with an acute abdomen due to CC rupture and biliary peritonitis. The reported incidence ranges between 2-18%^[11]. CC perforation is generally spontaneous, although sometimes trauma may be implicated. It is more frequent in children, while in adults it is associated with conditions causing increased intra-abdominal pressure, such as pregnancy^[12]. In the present case, no obvious cause of rupture was

identified, considering that no kind of abdominal pressure was applied during the MRI/MRCP procedure. We thus believe that rupture was an accidental event. The recommended acute therapy for CC rupture is peritoneal drainage, followed by complete cyst resection, cholecystectomy and Roux-en-Y hepaticojejunostomy reconstruction. Our patient was therefore treated primarily as a surgical emergency with peritoneal drainage and a more definitive surgical procedure was accordingly scheduled.

Controversy exists about the role of hepatic resection in types IV and V cysts due to the difficulty of intrahepatic cyst excision, thus rendering these patients to a more intense long-term follow-up. Alternatively, minimally invasive and laparoscopic treatments have been proposed^[9,13,14].

In conclusion, apart from the clinical evidence, the diagnostic algorithm in this case as well as postoperative evaluation comprises information acquired by a combination of imaging modalities, namely CT, MRI/MRCP, and hepatobiliary scintigraphy. The proper handling of such patients relies upon good co-operation between several medical faculties, namely gastroenterology, radiology, nuclear medicine, and surgery, and is essential in order to prevent and treat serious complications.

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Ultrastructure of Kupffer cells and hepatocytes in the Dubin-Johnson syndrome: A case report

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Abstract

Ultrastructure of Kupffer cells and hepatocytes in liver biopsate was evaluated in a 17-year-old boy with Dubin-Johnson syndrome (DJS). The liver tissue obtained by needle biopsy was fixed in glutaraldehyde and paraformaldehyde and routinely processed for electron microscopic analysis. The ultrastructural examinations of liver biopsate revealed the accumulation of membrane-bound, electron-dense lysosomal granules within the cytoplasm of hepatocytes, characteristic of DJS. They were located mainly in the vicinity of the biliary pole, and preferentially in the centrilobular region that corresponded to the pigment deposits seen under light microscope. The presence of the granules was accompanied by dilated elements of the granular endoplasmic reticulum and paracrystalline mitochondrial inclusions as well as dilation of the bile canaliculi. The changes in hepatocytes coexisted with marked stimulation and enhanced phagocytic activity of Kupffer cells. This was manifested in the accumulation of pigment deposits within their cytoplasm that corresponded to those observed in hepatocytes. Hyperactive pericentral Kupffer cells which are involved in the response to pigmentary material originating from disintegrated hepatocytes may play an essential role in the development of DJS.

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Keywords: Liver biopsy; Electron microscopic study; Lysosomal granules; Functional hyperbilirubinemia; Dubin-Johnson syndrome

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INTRODUCTION

A rise in serum bilirubin concentration above the norm may be caused by its increased production (hemolytic processes, shunt-bilirubinemia), impairment of its uptake, conjugation and discharge from hepatocytes (congenital non-hemolytic functional hyperbilirubinemia) or excretion of unconjugated and conjugated bilirubin from damaged hepatocytes or biliary canaliculi to the blood. Clinical picture can be similar, as jaundice is the predominant and often isolated symptom in the above pathologies. In doubtful cases, morphological examination of a liver biopsate is a decisive step^[1-3].

The main aim of the paper was to demonstrate the ultrastructure of Kupffer cells and hepatocytes in liver biopsy from a patient with Dubin-Johnson syndrome (DJS). To the best of our knowledge, it is the first report on the ultrastructure of Kupffer cells in this disorder.

CASE REPORT

Our report presents data concerning a 17-year-old boy suffering from hyperbilirubinemia (up to 119.7 $\mu\text{mol/L}$) with concomitant normal activity of aminotransferases from 6 years of age. He was suspected of congenital functional hyperbilirubinemia. However, since abdominal complaints and hepatomegaly were found to coexist with scleral xanthosis, the boy was sent for further diagnosis to the 3rd Department of Pediatrics, Medical University of Bialystok. On admission, laboratory tests revealed high serum bilirubin concentration (120.55 $\mu\text{mol/L}$) with the predominance of unconjugated fraction (82.94 $\mu\text{mol/L}$). However, the activities of aminotransferases (ALT, AST), alkaline phosphatase and GGTP were not increased. Differential diagnosis excluded: hemolytic disorders, infections with hepatotropic viruses (HBV, HCV, CMV, EBV, HSV), metabolic disorders (cystic fibrosis, Wilson's disease, α -1 antitrypsin deficiency, hemochromatosis), hypothyroidism, autoimmune liver diseases, parasitic and epizootic diseases. The ultrasonographic examination of the abdominal cavity confirmed hepatomegaly with no changes in parenchyma echogenicity. Liver biopsy was performed to elucidate the still unclear cause of jaundice.

For ultrastructural analysis, small, fresh tissue blocks (1 mm³) were cut off from the cylinder of liver tissue obtained by needle biopsy. The remaining considerable part of the collected tissue was submitted for examination by the light microscopy.

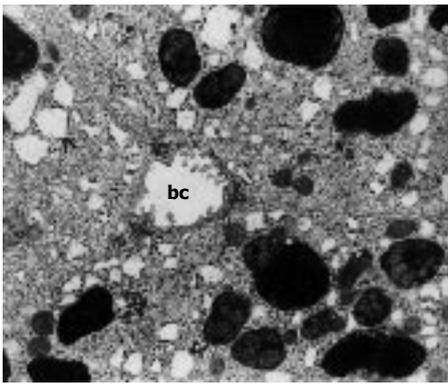


Figure 1 Several membrane-bound granules – varying in size, content, and electron density – located near the biliary pole of hepatocytes; broadening of granular endoplasmic reticulum (>); dilation of bile canaliculus (bc) with the loss of microvilli ($\times 7\ 000$).

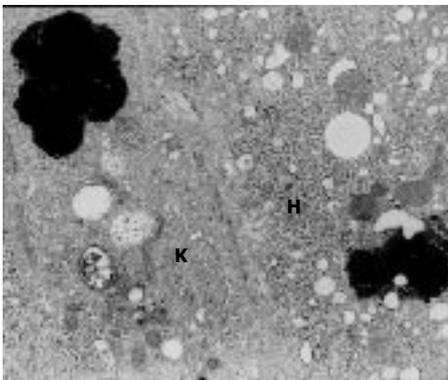


Figure 2 Fragment of Kupffer cell with phagocytized thick black pigment granules; the cell adheres to a hepatocyte (H) containing a similar pigment deposit ($\times 7\ 000$).

The material for electron microscopic analysis was fixed in 10 g/L glutaraldehyde and 8 g/L paraformaldehyde in 0.1 mol/L cacodylate buffer (pH 7.4). Subsequently, fixation was carried out in osmium tetroxide; then the material was routinely processed for embedding in Epon 812. Ultrathin sections were contrasted with uranyl acetate and lead citrate, and examined using an Opton 900 PC transmission electron microscope (Zeiss, Oberkochen, Germany).

The liver specimen obtained by needle biopsy was grossly dark and greenish, the striking characteristics for DJS. Liver histology showed numerous distinct coarse brownish pigment deposits in the cytoplasm of the hepatocytes.

Ultrastructural examination of these cells showed electron-dense, lysosomal granules, characteristic of DJS, that corresponded to the intracytoplasmic pigment deposits seen in the light microscopic sections. This pigmentary material was frequently surrounded by a single, fine continuous or discontinuous membrane; sometimes no such membrane could be found. The lysosomal pigment granules demonstrated great variability in shape, size, content, and electron density and were located mainly in the vicinity of the biliary pole (Figure 1), and preferentially found in the centrilobular region. Moderately

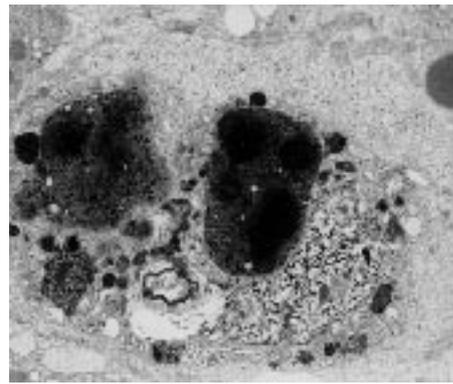


Figure 3 A hypertrophic, evidently activated Kupffer cell with phagocytized, very thick, black pigment deposits and biliary lamellar material. The cell blocks the sinusoidal vascular lumen. Distinctly dilated Disse's space contains a fine granular material ($\times 4\ 400$).

electron-dense finely granular background alternating with denser irregular areas and pleomorphic dark bodies (clearly seen against a finely homogenous granular matrix) characterized these granules. The pleomorphic very dense bodies frequently occupied a considerable part or almost whole lysosomes. Some hepatocytes filled with coarse electron-dense granules were disintegrated. Dilation of bile canaliculi with loss of microvilli typical for DJS was observed (Figure 1). Dilated elements of the granular endoplasmic reticulum (Figure 1), the presence of paracrystalline mitochondrial inclusions, enlarged Golgi complex and dispersed lipid droplets within the cytoplasm of hepatocytes were also noted. The vascular pole of the hepatocytes showed alteration of microvilli including their disintegration; a fine granular material could be occasionally seen in Disse's space. Sometimes this space was discontinued. The basement membrane of some hepatocytes was distinctly outlined, and even thickened.

The ultrastructural examinations of the liver biopate also showed, especially in the centrilobular region, an increase in hepatic macrophages. Kupffer cells in the lumen of hepatic sinusoidal vessels were frequently hypertrophied and markedly activated, blocking the vascular lumen and demonstrating features of hyperactivity (Figure 3). They contained electron-dense dark pigment granules, varying in shape and size (Figures 2 and 3), often occupying almost whole lysosomes. This material corresponded to the pigment deposits present in hepatocytes (Figure 2). Bile deposits were sometimes observed near the plentiful phagocytized pigment granules (Figure 3).

Since the morphological picture of the liver biopate was typical of DJS, leaving no doubt, we did not perform very expensive genetic tests (mutation in the *ABCC2/ MRP2* gene^[4]).

DISCUSSION

Macro- and microscopic, especially ultrastructural, changes observed in the liver biopate in our patient were characteristic of DJS. The hepatocytes contained numerous electron-dense lysosomal, membrane-bound granules located mainly near the biliary pole that corresponded to

the coarse brownish pigment deposits seen under light microscope. The granules were accompanied by dilated endoplasmic reticulum and paracrystalline inclusions as well as alterations in bile canaliculi of hepatocytes.

However, Kupffer cells demonstrated features of hyper-reactivity. They accumulated pigment granules that corresponded to those observed in hepatocytes.

Morphological abnormalities of hepatocytes observed in the course of our studies were similar to those found by other authors in this syndrome^[3,5-7]. It is worth noting that although the pigment granules showed characteristic microscopic features, their exact composition has not been clarified^[1,6]. The results of ultrastructural and histochemical studies by Luo *et al.*^[2] suggest that this deposit material is a lipofuscin–melanin complex.

Up to now, there have been no reports describing the electron microscope picture of Kupffer cells in DJS.

It has been known that Kupffer cells are the largest population of tissue macrophages, predominantly distributed in the lumen of hepatic sinusoids. According to Naito *et al.*^[8], these cells demonstrate unique differentiation mechanisms, metabolic functions and responsiveness to inflammatory agents. They exhibit endocytic activity against blood-borne material entering the liver as well as phagocytic function in relation to cellular debris^[8,9]. Kupffer cells also play an important role in the modulation of drug metabolic enzymes^[10], in the carcinogenesis of hepatocellular carcinoma^[11] and liver fibrosis^[12].

We assume that pigment deposits absorbed by Kupffer cells in the course of DJS may have originated from disintegrating, pigment-load hepatocytes. The present study indicates that it is the process of phagocytosis that plays a significant role in the accumulation of thick black granules within the cytoplasm of these macrophages. This process is strictly connected with the morphological status and function of lysosomes of Kupffer cells. This is confirmed by the fact that the number of lysosomes, both primary and secondary, within the cytoplasm of hyperactivated macrophages was markedly increased in our observations. It can be suggested that in the course of DJS, due to phagocytosis, remnants of pigment-load hepatocytes trapped by the hyperactivated Kupffer cells with the involved macrophage scavenger receptors are shifted to primary lysosomes, i.e. intracytoplasmic reservoirs containing lytic enzymes, which undergo degradation. Then, the lysosomes are transformed into the secondary ones^[8].

Unlike in the Gilbert's syndrome, in DJS clinical manifestation of hepatomegaly can be found even in the

neonatal-infantile period^[3,7,13-14]. In our patient, the cause of hyperbilirubinemia was unknown for many years. DJS was not taken into account because of atypical course of the disease (predominance of unconjugated bilirubin in the blood serum). However, the predominance of non-conjugated bilirubin in the blood serum has been noted in some diagnosed DJS cases^[1].

We emphasize that hyperactive pericentral Kupffer cells may play an essential role in the development of the syndrome. They are involved in the response to pigmentary material originating from disintegrated hepatocytes.

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CASE REPORT

Pyogenic liver abscess associated with large colonic tubulovillous adenoma

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Abstract

Pyogenic liver abscesses usually occur in association with a variety of diseases. Rarely, liver abscess has been reported as the presenting manifestation of colonic tubulovillous adenoma. We report two cases of pyogenic liver abscess without hepatobiliary disease or other obvious etiologies except that one had a history of diabetes mellitus (DM). The pathogen in the patient with DM was *Klebsiella pneumonia* (KP). In both of the patients, ileus developed about two to three weeks after the diagnosis of liver abscess. Colonoscopy revealed large polypoid tumors with pathological findings of tubulovillous adenoma in both cases. Two lessons were learned from these two cases: (1) an underlying cause should be aggressively investigated in patients with cryptogenic liver abscess; (2) DM could be one of the etiologies but not necessarily the only cause of KP liver abscess.

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Key words: Pyogenic liver abscess; *Klebsiella pneumonia*; Colonic tubulovillous adenoma

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INTRODUCTION

Pyogenic liver abscess is usually related to hepatobiliary

tract disease or intra-abdominal infections including cholecystitis, suppurative cholangitis, suppurative pyelophlebitis, appendicitis, diverticulitis and peritonitis^[1-3]. Recently, colorectal cancer without obvious infection was considered as one of the uncommon etiologies of liver abscess^[4,5]. Furthermore, liver abscess was reported as the presenting manifestation of colonic tubulovillous adenoma^[6]. However, there are a growing number of reports suggesting a relationship between diabetes mellitus (DM) and *Klebsiella pneumonia* (KP) liver abscess which indicate that a deficiency in the hosts' defense mechanism could be the etiology of this liver infection^[7-9]. We hereby report two cases of pyogenic liver abscess both of which were found to have colonic tubulovillous adenoma two to three weeks later after the diagnosis of liver abscess. The association of pyogenic liver abscess and colonic tubulovillous adenoma and the implications of these two cases are discussed.

CASE REPORT

CASE 1

A 67-year-old man, who was referred from a local hospital, was admitted to our hospital because of fever, chill, and right upper abdominal pain for seven days. On physical examination, his initial body temperature was 37.9°C and blood pressure was 124/67 mmHg. Light palpation of the abdomen revealed tenderness and mild rebounding pain on epigastric and right upper areas, but no abdominal mass was felt. Significant laboratory data included a leukocyte count of 26,330/ μ L (normal range = 4000-10,400/ μ L), a hemoglobin of 9.5 gm/dL (normal range = 12-16 gm/dL), an aspartate aminotransferase (AST) of 73 IU/L (normal range = 5-34 IU/L), an alanine aminotransferase (ALT) of 79 IU/L (normal range = 0-40 IU/L), a fasting blood sugar of 118 mg/dl (normal range = 70-110 mg/dL), a total bilirubin of 1.03 mg/dL (normal range = 0.2-1.3 mg/dL), a C-Reactive Protein (CRP) of 17.02 mg/dL (normal <0.8 mg/dL), and a carcinoembryonic antigen (CEA) level of 0.33 ng/dL (normal <5 ng/dL).

Abdominal ultrasonography (US) and computed tomography (CT) showed a 4-cm liver abscess in left lobe of liver (Figure 1A). After three sets of blood cultures were completed and broad-spectrum antimicrobial therapy was initiated, CT-guided percutaneous drainage of the liver abscess was performed and pus like material was aspirated and sent for bacterial culture. However, blood and pus were all negative for bacterial growth. As antimicrobial treatment was continued, the patient's clinical condition improved rapidly and fever subsided four days

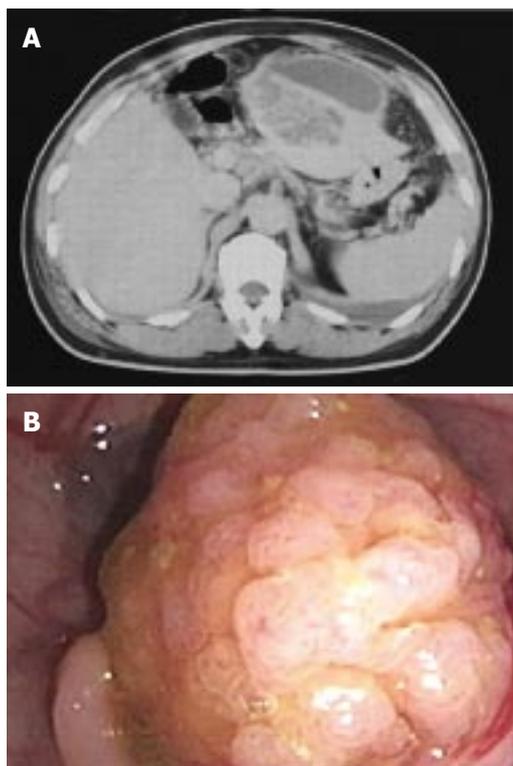


Figure 1 Case 1. **A:** Enhancement abdominal computed tomography (CT) showed a 4-cm liver abscess in left lobe of the liver; **B:** Colonofibroscopy showed a 3-cm pedunculated polyp in the sigmoid colon.

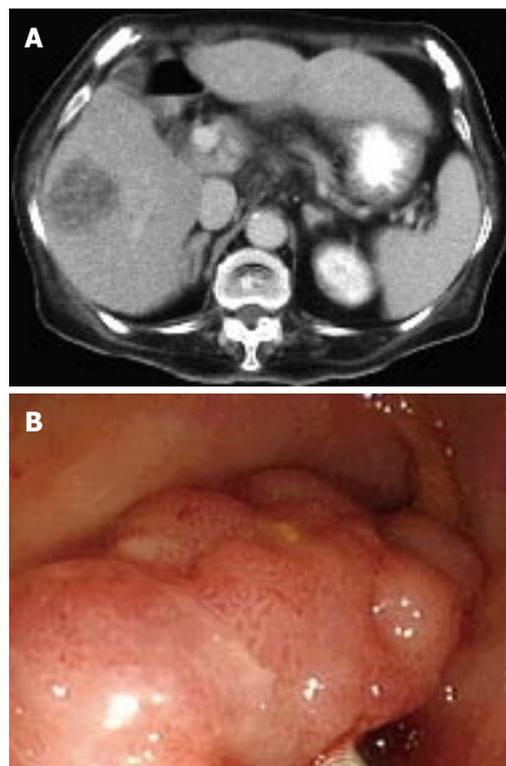


Figure 2 Case 2. **A:** Enhancement abdominal computed tomography showed a 5-cm liver abscess in right lobe of the liver; **B:** Colonofibroscopy showed a 3-cm polypoid tumor in the sigmoid colon.

later. Then the drainage catheters were removed and the patient was discharged on the 14th d of hospitalization.

One week later, he was admitted again with a chief complaint of abdominal fullness. Physical examination showed a distended abdomen with hypertympanic abdominal sounds on percussion. Plain films of the abdomen disclosed evidence of a paralytic ileus with a suspected obstruction at the level of the sigmoid colon. Colonoscopy was performed which showed a 3-cm pedunculated polyp in the sigmoid colon (Figure 1B). Polypectomy was performed and pathological examination revealed tubulovillous adenoma with mild dysplasia. The patient continued to do well during a follow-up period of 6 mo.

CASE 2

An 84-year-old woman with a history of DM using no regular medication for several years was admitted to the hospital because of fever and right upper abdominal dull pain which was followed by a change of consciousness for the two days preceding admission. On physical examination, her initial body temperature was 37.7°C and blood pressure was 79/30 mmHg. Mild tenderness in the right upper abdomen was noted during performance of light palpation, but no abdominal mass was felt. Significant laboratory data included a leukocyte count of 17,870/ μ L, a hemoglobin of 12.7 mg/dL, an AST of 59 IU/L, an ALT of 58 IU/L, a fasting blood sugar of 348 mg/dL, a total bilirubin of 2.31 mg/dL, a CRP level of 32.82 mg/dL, and a CEA level of 6.72 ng/dL. Abdominal US and CT showed a 5-cm liver abscess in right lobe of the liver (Figure 2A).

Since the patient could not cooperate for US or CT-guided aspiration, broad-spectrum antimicrobial treatment was started after three sets of blood cultures were performed. The results of blood cultures were positive for *Klebsiella pneumoniae* (KP), which was sensitive to the formerly prescribed antibiotics. The patient's clinical condition improved gradually and fever subsided 10 d later. On the 14th d of hospitalization, abdominal fullness developed and the plain film of abdomen showed evidence of ileus with a suspected obstruction at the level of the distal colon. Colonoscopy was performed and showed a 3-cm polypoid tumor in the sigmoid colon (Figure 2B). Polypectomy was not done because of technical difficulty. A biopsy was performed and eight pieces of tissue were taken for pathological examination. The pathological findings were consistent with tubulovillous adenoma. Since ileus resolved spontaneously and the patient's general physical condition was not good, the patient's family refused to have further treatment. Her general condition was **stable** during a follow-up period of two months.

DISCUSSION

The etiologies and pathogenesis of liver abscesses have changed significantly over the past several decades^[1-3,10-11]. Recent reports suggest that pyogenic liver abscess might be a warning indicator of silent colonic cancer^[4,5,12-14], therefore, patients with liver abscess but without apparent etiologies, as in Case 1 of this report, should have further examination. In addition, non-growth in cultures of blood samples and abscess aspirate in Case 1 could be due to

the following two possibilities: (1) the patient had been partially treated at an outside hospital; (2) the pathogen of the liver abscess might be an anaerobe that was most probably originated from the colon. Therefore, although a rapid improvement was noted, a complete evaluation of the GI tract should be performed in Case 1 as per the suggestion of Cohen *et al*^[4].

Nevertheless, colonoscopy was performed because of ileus and the examination revealed a large colonic tubulovillous adenoma in the sigmoid colon instead of a "silent colonic cancer". Whether colonic adenoma could be an etiology of pyogenic liver abscess remains to be elucidated. In addition to our report, there is only one other report of pyogenic liver abscess as the presentation of colonic villous adenoma^[6]. The mechanism for explaining colonic adenoma as an etiology of liver abscess may be the same as that of the colonic cancer, i.e., there are mucosal defects present on the colonic lesions (cancers or adenomas) that allow a route for bacteria invasion into the portal system with subsequent hematogenous spread to the liver.

In recent years, pyogenic liver abscesses have occurred more frequently in immunodeficient patients, including those receiving intensive chemotherapy for hematological or solid malignancies, patients with immunosuppressive therapy after organ transplantation^[15] and, especially, patients with DM^[8-9]. Case 2 was a DM patient who had a solitary liver abscess caused by KP. As previous reports showed, KP liver abscesses tend to occur in DM patients who did not have any predisposing factors for the development of liver abscess, therefore, the diagnostic procedures and management in Case 2 seemed reasonable. However, the unexpected occurrence of ileus due to a large colonic villous adenoma indicated that the presence of DM could not completely exclude other possible etiologies of liver abscess.

In conclusion, for patients with cryptogenic liver abscess, an underlying cause should be aggressively searched for. DM could be one of the etiologies but not necessarily the only cause of KP liver abscess.

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Delayed ischemic gangrene change of distal limb despite optimal decompressed colostomy constructed in obstructed sigmoid colon cancer: A case report

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decompressed colostomy would resolve acute malignant colon obstruction efficiently; impending ischemic bowel may progress with a possible irreversible peritonitis. Any patient, who undergoes a decompressed colostomy without resection of the obstructed lesion, should be monitored with leukocyte count and abdominal condition survey frequently.

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Key words: Colorectal cancer obstruction; Colostomy; Ischemic colitis; Laparotomy

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Abstract

Creating blow-hole colostomy for decompression could provide a time-saving and efficient surgical procedure for a severely debilitated case with a completely obstructed colorectal cancer. Complications are reported as prolapse, retraction, and paracolostomal abscess. However, complication with an ischemic distal limb has not been reported. We report a case of critical intra-abdominal disease after decompressed colostomy for relieving malignant sigmoid colon obstruction; a potential fatal condition should be alerted. A 76-year-old male visited our emergency department for symptoms related to obstructed sigmoid colon tumor with foul-odor vomitus containing fecal-like materials. An emergent blow-hole colostomy proximal to an obstructed sigmoid lesion was created, and resolution of complete colon obstruction was pursued. Unfortunately, extensive abdominal painful distention with board-like abdomen and sudden onset of high fever with leukocytopenia developed subsequently. Such surgical abdomen rendered a secondary laparotomy with resection of the sigmoid tumor along with an ischemic colon segment located proximally up to the previously created colostomy. Eventually, the patient had an uneventful postoperative hospital stay. In the present article, we have described an emergent condition of sudden onset of distal limb ischemia after blow-hole colostomy and concluded that despite the

INTRODUCTION

In 8-29% of patients with colorectal cancer, obstruction is the major symptom at presentation, and 85% of patients undergoing emergency colorectal surgery have complete colonic obstruction from colorectal carcinoma^[1-4]. Obstructed colorectal cancer often dictates poor cancer-related prognosis and higher mortality or morbidity, because they are more advanced and are associated with a higher incidence of distant metastases^[3,5]. One-stage surgery is believed to be the preferred procedure for an obstructive right colon lesion, and staged operation is favored for the management of malignant left-side colonic obstructions^[6]. In patients with acute complete colonic obstruction, hemodynamic instability may be debilitated by concomitant medical diseases, and cancer resection may not be possible. Establishing a decompressed colostomy is usually recommended to resolve acute obstructive symptoms prior to cancer resection^[7,8].

Obstructive colorectal cancer may be complicated with concomitant proximal ischemic colitis, which would present dramatically with gangrene and/or colonic perforation, if the acute vascular insufficiency is severe^[9]. In general, colonic decompression by way of self-expanding metal stent or decompressed colostomy could

deliver intraluminal pressure lower enough than intramural vascular perfusion pressure to avoid hypoperfusive status of the intestine proximal to obstructive lesion. It is common to manifest proximal colon distention in left-sided colorectal cancer obstruction if a competent ileocecal valve exists^[10], and impending ischemic change of proximal colon would pursue. No fatal intra-abdominal complication for constructing a blow-hole colostomy, such as a gangrenous distal limb with peritonitis, has been reported.

We here report a case of severe intra-abdominal condition despite that a functional decompressed colostomy was created, a potential fatal situation of which the surgeons should keep in mind when relieving malignant colon obstruction using blow-hole colostomy.

CASE REPORT

A 76-year-old male patient, who had extensive abdominal distention associated with no stools or flatus passage for 2 d, was referred to our emergency department for sudden onset of vomiting with foul-odor and fecal material-like contents. He had a 4-mo history of gradual body weight loss, abdominal bloat, bowel habit change with decreased stool caliber and poor appetite. Abdominal computerized topography revealed an obstructive sigmoid colon lesion with segmental wall thickening and a stenotic lumen. There was a pill within the distended bowel segment immediately proximal to the stenotic lumen of the obstructed lesion. Sigmoid colon tumor with complete bowel obstruction was diagnosed based on history and image studies. The patient had underlying chronic obstructive pulmonary disease with intermittent consumption of inhalational bronchodilator. Physical examinations reviewed a distended abdomen with silent bowel sound; there was tachypnea with central bronchospasm. Hemogram showed WBC as 11 200/ μL with a differential count of segment form, 78.4%; band form, 1%; and lymphocyte, 10.8%. Biochemical analysis showed slightly impaired renal function (BUN: 31 mg/dL, Cr: 2.1 mg/dL), normal electrolyte concentration (Na: 136 meq/L, K: 4.0 meq/L and Cl: 103 meq/L) and suboptimal nutritional status (albumin: 2.6 g/dL). Clinically, the patient complained of serious abdominal painful fullness, fever with chills, and dyspnea. Metal stenting to the colon stenotic lesion was attempted but failed. On account of emergent release of the obstructive bowel pressure, the patient underwent a blow-hole colostomy at proximal transverse colon by mini-laparotomy, which took less than 30 min under general anesthesia. The bowel segment at the operation field was severely distended with edema, however, adequate blood perfusion was observed. There was no ascites. A large amount of liquid yellowish stools with foul smell was drained out from the proximal and distal limbs of the colostomy after it was instituted. The patient sustained a smooth operation course.

A rechecked hemogram at the 2nd post-operation day, which is routinely given to patients undergoing abdominal surgery in our institute as a baseline for follow-up purpose, found rather unremarkable data (WBC as 8 800/ μL with slightly elevated segment form of 84%, without band form).

Gradual remission of abdominal distention with persistent bowel content drainage was observed within the first three post-operation days; the colostomy would be deemed as a normal physical function. Unfortunately, extensive abdominal painful distention with board-like abdominal appearance, and sudden onset of high fever with a body temperature up to 39.8°C were noted afterwards. At follow-up, hemogram reviewed leukocytopenia with dominant left-shifted differential count (WBC: 1 600/ μL , segment: 38%, band: 26%). We found such a surgical abdomen that an emergent operation should be done to explore the etiology.

Emergent laparotomy was done immediately to find marked foul-odor bloody ascites. The distal limb of colostomy extending from the colostomy distally to the obstructed sigmoid colon tumor was found to have severe ischemic gangrenous change. The proximal limb of colostomy was fine. Segmental resection of the lesioned colon, comprising the sigmoid colon cancer with a security of cancer-free distal section margin and a short segment of colon proximal to previously created colostomy, was performed. The blow-hole colostomy was then converted to end-T-colostomy. The patient exerted an uneventful postoperative course and scheduled colostomy reversal was undertaken 6 mo later.

DISCUSSION

Fine construction of a transverse colostomy by drawing out of a loop of the bowel and securing the mesentery to the skin to decompress obstructions from carcinoma of the rectum was first described in 1797^[11]. Intestinal obstruction from malignancy within the colorectum would carry catastrophic complications of perforation, electrolyte imbalance, bowel ischemia, and sepsis caused by bacterial translocation^[12]. Moreover, cancer patients, especially those with bowel obstruction, are usually malnourished, older, and dehydrated and may have coexisting disease at the time of presentation^[12-14]. Therefore, perioperative management of malignant bowel obstruction, inclusive of surgical planning option to minimize morbidity and mortality is a critical issue.

There are different strategies to decompress colon obstruction with different outcomes, including (1) non-surgical approach as balloon dilation, placement of a plastic non-expandable rectal tube, cryosurgical destruction, electrocoagulation, and laser ablation^[15,16] and (2) surgical approach as blow-hole enterostomy, or resection of tumor-bearing colorectal segment with or without colostomy^[2,4-8]. Because of the failure of self-expanding metal stent placement in this patient due to severe colon angulations and rather stenotic colon lumen, we recommended a blow-hole proximal transverse colostomy as an emergent procedure for this debilitated patient with severe coexisting medical condition.

To evaluate the surgical planning in malignant large bowel obstruction, it is widely accepted that for malignant colon obstruction from the right colon cancer, resection (right hemicolectomy) should be performed with anastomosis in one stage^[5,17,18]. Nevertheless, for obstruction from cancer of left colon or rectum, two types

of surgical approach could be used: (1) primary resection and primary anastomosis or Hartmann's procedure with synchronous treatment of carcinoma and obstruction, and (2) staged resection with the treatment of the obstruction prior to resection^[4]. Some surgeons prefer staged resection with fast resolution of the obstruction, and a delayed radical surgery. The main benefits of staged operation are less surgical trauma which is significant in patients whose general condition is precarious, and reduction of the risk of contamination due to unprepared bowel^[19]. Poor physical status as this patient, construction of a blow-hole colostomy in a minimal wound (fit to colostomy size) with less time-consumption (less than 30 min) could offer an anticipated decompression effect of the obstruction and prevent further surgical trauma to facilitate scheduled radical resection in the future.

Ischemic episode would take place in malignant large bowel obstruction and present dramatically with gangrene and/or colonic perforation if acute vascular insufficiency is severe^[9]. It is postulated that colon distention from malignant colon obstruction reduces the blood supply to the bowel wall and enhances the development of ischemia^[10]. Creation of a decompressed colostomy in malignant colon obstruction should rescue impending elevated intra-luminal pressure and possible development of the proximal colitis. But in our case, the cause of gangrenous distal limb might be due to, first, the development of tumor thrombi or septic emboli in the territory of vessels supplying the distal colon, or second, an underlying undetected ischemic change proximal to the cancerous mass which was not observed for the initial limited surgical field by mini-laparotomy. Clinically, the patient sustained sudden onset of high fever, peritoneal signs, and leukocytopenia with dominant left-shifted differential count on the 4th post-operation day, which was in the opposite direction to recovering status. Although, toxic signs presented by this patient alerted us of his deteriorated status, ischemic gangrene change of the distal limb between created stoma and obstructed sigmoid colon cancer was beyond our expectation. Nevertheless, bloody mucoid stools, which are usually in consistent with the clinical features of ischemic colitis, was not identified from the distal stoma ahead of the time in which toxic signs presented.

In conclusion, impending ischemic bowel may progress with a catastrophic peritonitis despite construction of blow-hole colostomy, which rescued malignant sigmoid colon obstruction. Consequently, it is important to monitor leukocyte count change and survey meticulously

abdominal condition for patients with decompressed colostomy created without resection of the obstructed lesion.

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Meetings

MAJOR MEETINGS COMING UP

Digestive Disease Week
107th Annual Meeting of AGA, The American
Gastroenterology Association
May 20-25, 2006
Loas Angeles Convernition Center, California
www.ddw.org

EVENTS AND MEETINGS IN THE UPCOMING 6 MONTHS

10 th World Congress of the International Society
for Diseases of the Esophagus (ISDE 2006)
February 22-25, 2006
Adelaide
isde@sapmea.asn.au
www.isde.net

EASL 2006 - The 41st Annual Meeting
April 26-30, 2006
Vienna, Austria

International Gastrointestinal Fellows Initiative
February 22-24, 2006
Banff, Alberta
CAGOffice@cag-acg.org
www.cag-acg.org

Canadian Digestive Disease Week
February 24-27, 2006
Banff, Alberta
CAGOffice@cag-acg.org
www.cag-acg.org

European Multidisciplinary Colorectal Cancer
Congress 2006
February 12-14, 2006
Berlin
info@congresscare.com
www.colorectal2006.org

ILTS 12th Annual International Congress
May 3-6, 2006
Milan
www.ilsts.org

World Congress on Gastrointestinal Cancer
June 14-17, 2006
Barcelona, Spain
c.chase@imedex.com

5th International Congress of The African Middle
East Association of Gastroenterology
February 24-26, 2006
Sharjah
infoevent@infomedweb.com
www.infomedweb.com

Digestive Disease Week 2006
May 20-25, 2006
Los Angeles
www.ddw.org

Annual Postgraduate Course
May 25-26, 2006
Los Angeles, CA
www.asge.org/education

EVENTS AND MEETINGS IN 2006

10th World Congress of the International Society
for Diseases of the Esophagus (ISDE 2006)
February 22-25, 2006
Adelaide
isde@sapmea.asn.au
www.isde.net

10th International Congress of Obesity
September 3-8, 2006
Sydney
enquiries@ico2006.com
www.ico2006.com

EASL 2006 - The 41st Annual Meeting
April 26-30, 2006
Vienna, Austria

International Gastrointestinal Fellows Initiative
February 22-24, 2006
Banff, Alberta
CAGOffice@cag-acg.org
www.cag-acg.org

Canadian Digestive Disease Week
February 24-27, 2006
Banff, Alberta
CAGOffice@cag-acg.org
www.cag-acg.org

Prague Hepatology Meeting 2006
September 14-16, 2006
Prague
veronika.revicka@congressprague.cz
www.czech-hepatology.cz/phm2006

European Multidisciplinary Colorectal Cancer
Congress 2006
February 12-14, 2006
Berlin
info@congresscare.com
www.colorectal2006.org

World Congress on Controversies in Obesity,
Diabetes and Hypertension (CODHy)
October 25-28, 2006
Berlin
codhy@codhy.com
www.codhy.com

ILTS 12th Annual International Congress
May 3-6, 2006
Milan
www.ilsts.org

XXX pan-american congress of digestive diseases
November 25-December 1, 2006
Cancun
amg@gastro.org.mx
www.gastro.org.mx

World Congress on Gastrointestinal Cancer
June 14-17, 2006
Barcelona, Spain
c.chase@imedex.com

5th International Congress of the African Middle
East Association of Gastroenterology
February 24-26, 2006
Sharjah
infoevent@infomedweb.com
www.infomedweb.com

7th World Congress of the International Hepato-
Pancreato-Biliary Association
September 3-7, 2006
Edinburgh
convention@edinburgh.org
www.edinburgh.org/conference

Digestive Disease Week 2006
May 20-25, 2006
Los Angeles
www.ddw.org

Annual Postgraduate Course
May 25-26, 2006
Los Angeles, CA
www.asge.org/education

71st ACG Annual Scientific Meeting and
Postgraduate Course
October 20-25, 2006
Venetian Hotel, Las Vegas, Nevada

AASLD 57th Annual Meeting - The Liver Meeting™
October 27-31, 2006
Boston, MA

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- 3 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaobua Zazhi* 1999; 7: 285-287

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- 4 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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- 5 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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- 6 **Morse SS**. Factors in the emergence of infectious diseases. *Emerg Infect Dis* serial online, 1995-01-03, cited 1996-06-05; 1(1):24 screens. Available from: URL: <http://www.cdc.gov/ncidod/EID/eid.htm>

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Present as mean \pm SD and mean \pm SE.

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Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as γ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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Use SI units. For example: body mass, *m* (B) = 78 kg; blood pressure, *p*(B) = 16.2/12.3 kPa; incubation time, *t* (incubation) = 96 h, blood glucose concentration, *c* (glucose) 6.4 \pm 2.1 mmol/L; blood CEA mass concentration, *p* (CEA) = 8.6 24.5 μ g/L; CO₂ volume fraction, 50 mL/L CO₂ not 5% CO₂; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, etc. Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

Genotypes: *gyrA*, *arg 1*, *c myc*, *c fos*, etc.

Restriction enzymes: *EcoRI*, *HindI*, *BamHI*, *Kho I*, *Kpn I*, etc.

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World Journal of Gastroenterology standard of quantities and units

| Number | Nonstandard | Standard | Notice |
|--------|--|--|--|
| 1 | 4 days | 4 d | In figures, tables and numerical narration |
| 2 | 4 days | four days | In text narration |
| 3 | day | d | After Arabic numerals |
| 4 | Four d | Four days | At the beginning of a sentence |
| 5 | 2 hours | 2 h | After Arabic numerals |
| 6 | 2 hs | 2 h | After Arabic numerals |
| 7 | hr, hrs, | h | After Arabic numerals |
| 8 | 10 seconds | 10 s | After Arabic numerals |
| 9 | 10 year | 10 years | In text narration |
| 10 | Ten yr | Ten years | At the beginning of a sentence |
| 11 | 0,1,2 years | 0,1,2 yr | In figures and tables |
| 12 | 0,1,2 year | 0,1,2 yr | In figures and tables |
| 13 | 4 weeks | 4 wk | |
| 14 | Four wk | Four weeks | At the beginning of a sentence |
| 15 | 2 months | 2 mo | In figures and tables |
| 16 | Two mo | Two months | At the beginning of a sentence |
| 17 | 10 minutes | 10 min | |
| 18 | Ten min | Ten minutes | At the beginning of a sentence |
| 19 | 50% (V/V) | 500 mL/L | |
| 20 | 50% (m/V) | 500 g/L | |
| 21 | 1 M | 1 mol/L | |
| 22 | 10 μM | 10 μmol/L | |
| 23 | 1N HCl | 1 mol/L HCl | |
| 24 | 1N H ₂ SO ₄ | 0.5 mol/L H ₂ SO ₄ | |
| 25 | 4rd edition | 4 th edition | |
| 26 | 15 year experience | 15- year experience | |
| 27 | 18.5 kDa | 18.5 ku, 18 500u or M:18 500 | |
| 28 | 25 g.kg ⁻¹ /d ⁻¹ | 25 g/(kg·d) or 25 g/kg per day | |
| 29 | 6900 | 6 900 | |
| 30 | 1000 rpm | 1 000 r/min | |
| 31 | sec | s | After Arabic numerals |
| 32 | 1 pg L ⁻¹ | 1 pg/L | |
| 33 | 10 kilograms | 10 kg | |
| 34 | 13 000 rpm | 13 000 g | High speed; g should be in italic and suitable conversion. |
| 35 | 1000 g | 1 000 r/min | Low speed. g cannot be used. |
| 36 | Gene bank | GenBank | International classified genetic materials collection bank |
| 37 | Ten L | Ten liters | At the beginning of a sentence |
| 38 | Ten mL | Ten milliliters | At the beginning of a sentence |
| 39 | umol | μmol | |
| 40 | 30 sec | 30 s | |
| 41 | 1 g/dl | 10 g/L | 10-fold conversion |
| 42 | OD ₂₆₀ | A ₂₆₀ | "OD" has been abandoned. |
| 43 | One g/L | One microgram per liter | At the beginning of a sentence |
| 44 | A260 nm ^b P<0.05 | A ₂₆₀ nm ^a P<0.05 | A should be in italic. In Table, no note is needed if there is no significance instatistics: ^a P<0.05, ^b P<0.01 (no note if P>0.05). If ther is a second set of P value in the same table, ^c P<0.05 and ^d P<0.01 are used for a third set: ^a P<0.05, ⁱ P<0.01. |
| 45 | [*] F=9.87, [§] F=25.9, [#] F=67.4 | ¹ F=9.87, ² F=25.9, ³ F=67.4 | Notices in or under a table |
| 46 | KM | km | kilometer |
| 47 | CM | cm | centimeter |
| 48 | MM | mm | millimeter |
| 49 | Kg, KG | kg | kilogram |
| 50 | Gm, gr | g | gram |
| 51 | nt | N | newton |
| 52 | l | L | liter |
| 53 | db | dB | decibel |
| 54 | rpm | r/min | rotation per minute |
| 55 | bq | Bq | becquerel, a unit symbol |
| 56 | amp | A | ampere |
| 57 | coul | C | coulomb |
| 58 | HZ | Hz | |
| 59 | w | W | watt |
| 60 | KPa | kPa | kilo-pascal |
| 61 | p | Pa | pascal |
| 62 | ev | EV | volt (electronic unit) |
| 63 | Jonle | J | joule |
| 64 | J/mmol | kJ/mol | kilojoule per mole |
| 65 | 10×10×10cm ³ | 10 cm×10 cm×10 cm | |
| 66 | N·km | KN·m | moment |
| 67 | x±s | mean±SD | In figures, tables or text narration |
| 68 | Mean±SEM | mean±SE | In figures, tables or text narration |
| 69 | im | im | intramuscular injection |
| 70 | iv | iv | intravenous injection |
| 71 | Wang et al | Wang <i>et al.</i> | |
| 72 | EcoRI | EcoRI | <i>Eco</i> in italic and RI in positive. Restriction endonuclease has its prescript form of writing. |
| 73 | Ecoli | <i>E.coli</i> | Bacteria and other biologic terms have their specific expression. |
| 74 | Hp | <i>H pylori</i> | |
| 75 | Iga | Iga | writing form of genes |
| 76 | igA | IgA | writing form of proteins |
| 77 | ~70 kDa | ~70 ku | |

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The role of ultrasonography in patients with celiac disease

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Abstract

The aim of the present review was to summarize the current evidence on the role of ultrasonography (US) and doppler-US in the diagnosis of celiac disease.

Several ultrasonographic signs have been reported in the association with celiac disease in studies using real-time US. Firstly, case control studies identified some of these US signs and then in a prospective series some of these parameters, due to their high specificity, have been shown to be of value in confirming CD diagnosis, whereas others, due to their high sensitivity, have been demonstrated to be useful in excluding the presence of the disease.

The pattern of splanchnic circulation in CD have extensively been investigated by several studies all of which reported similar results and identified a hyperdynamic mesenteric circulation that reverts to normal values after successful a gluten-free regimen.

The last part of this review will deal with the possible role of US in identifying the most severe and common intestinal complication of CD, i.e. the enteropathy-associated T cell non-Hodgkin lymphoma.

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Key words: Ultrasonography; Celiac disease

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INTRODUCTION

Celiac disease (CD) is a chronic systemic autoimmune disorder, whose prevalence in the general population ranges from 0.2 to 0.94^[1]. It occurs when the small bowel mucosa

of susceptible subjects is damaged by dietary gluten, and its clinical picture ranges from mild to severe forms. The most frequent findings include chronic diarrhea, iron-deficiency anemia and dyspepsia. The diagnosis is supported by the determination of endomysial (EmA) and transglutaminase (tTG) antibodies, characterized by a high sensitivity and specificity (> 85% and nearly 100%, respectively) and confirmed by consistent histological duodenal findings^[2]. Clinical, serological and histologic characteristics have recently been extensively reviewed^[1].

As reported above, clinical manifestations are proteiform and complications include refractory sprue, enteropathy-associated-T-cell-lymphomas (EATL), carcinoma of the oropharynx, esophagus and small bowel, ulcerative jejuno-ileitis and collagenous sprue^[3-7].

In patients with gastrointestinal symptoms abdominal ultrasonography (US) represents the initial imaging technique, whose accuracy has recently been markedly improved by the widespread availability of high resolution equipment. In this context several ultrasonographic signs have recently been reported^[8-12] and some of them have been proved to be of value in supporting or ruling out CD^[13]. This review deals with the role of abdominal and doppler ultrasonography in detecting the presence of celiac disease and in assessing its most frequent complication, i.e. EATL.

ABDOMINAL ULTRASOUND IN THE DIAGNOSIS OF CELIAC DISEASE

Different US signs have been proved to be of value in suggesting the presence of CD, case reports^[8-10] and two preliminary retrospective case-control studies, done in a pediatric^[11] or adult setting^[12] respectively. In the former series by Riccabona *et al*^[11], which included 39 children with overt malabsorption and histologically proven CD (Table 1), an "abnormal" aspect of small intestine was observed in 36 cases (92%), consisting of increased peristalsis in 32 (82%) and free abdominal fluid in 30 cases (76%); interestingly, a pericardial effusion was observed in half of the cases. Based on the above findings the Authors concluded that even if intestinal biopsy still remains the reference standard for diagnosing CD, the awareness of CD-associated sonographic abnormalities could accelerate diagnostic work-up and the introduction of gluten-free diet (GFD). The second study, by Rettenbacher *et al*^[12], included 11 adults with histologically proven CD and 20 healthy subjects (Table 2). An increase in intraluminal fluid content (11 cases), the presence of a moderate small bowel

Table 1 Number of patients showing a positivity of each of the four US signs studied in the pediatric series of Riccabona *et al*⁽¹¹⁾.

| US signs | Patients with a positive sign (#) |
|------------------------------------|-----------------------------------|
| Abnormal aspect of small intestine | 36 |
| Increased peristalsis | 32 |
| Abdominal fluid | 30 |
| Pericardial effusion | 18 |

Table 2 Number of patients showing a positivity for each of the eight US parameters assessed in the adult series of Rettenbacher *et al*⁽¹²⁾.

| US signs | Patients with a positive signs (#) |
|--------------------------------------|------------------------------------|
| Moderate small bowel dilatation | 8/11 |
| Increased intraluminal fluid content | 11/11 |
| Small bowel wall thickness | 7/11 |
| Increased peristalsis | 8/11 |
| Mesenteric lymph node hypertrophy | 9/11 |
| Free abdominal fluid | 5/11 |
| SMA or portal vein dilatation | 7/11 |
| Hepatic steatosis | 6/11 |

dilatation (8 cases) and of an increased peristalsis (8 cases), and the presence of moderate bowel wall thickness (7 cases) represented the most relevant findings. Other extra intestinal signs, as mesenteric lymph node enlargement, free abdominal fluid, superior mesenteric artery or portal vein dilatation, hepatic steatosis were also identified, with an overall frequency ranging from 52 to 84%. None of the healthy controls showed abnormal US signs.

Both the above series were aimed to answer a phase I question (i.e. are the results of the index test in patients with a target disorder different from those in controls?). These type of studies have the advantage of being relatively quick and cheap to perform and serve as a promoter for further studies; however they could be affected by the presence of a possible "spectrum bias" as only patients with fully expressed disease are usually studied^[14].

As a consequence, these types of studies should be confirmed in larger prospective cohorts to obtain both higher precision and wider generalizability.

In a recent study^[13], we have prospectively evaluated the diagnostic accuracy of different US signs in predicting CD in a cohort of 162 consecutive patients investigated for chronic diarrhea (# 105), iron-deficiency anemia (# 25) and dyspepsia (# 32) (i.e. frequent CD manifestations), and characterized by a pre-test probability of the disease ranging from 5 to 10% as estimated from a previous series^[15]. All patients underwent anti-endomysial IgA antibodies determination and duodenal biopsy. Two operators, blind to both the clinical, serological and histological findings evaluated six US signs represented by fasting gallbladder volume, transverse diameter of small bowel loops, small bowel wall thickness, peristaltic pattern, presence of free abdominal fluid, and diameter of the mesenteric lymph nodes.

Twelve patients (7%): six male and six female, aged 16-77 years with a mean of $49 \pm SD 17$ years, had EmA positive and duodenal histologic findings consistent with Marsh's grade III -IV (11 and one cases, respectively). In detail, seven cases were observed among those with chronic diarrhea (6.7%), four among those with iron-deficiency anemia (16%) and one among dyspeptics (3.1%). The frequency of the above mentioned US findings was comparable in pauci-symptomatic celiacs and those with fully expressed disease. Of the 150 EmA negative cases, none had duodenal histology consistent with CD; 72 (48%) and 78 (52%), were eventually diagnosed as having functional or organic disease, respectively. In the latter group, an ileal involvement was observed in nine cases (six with Crohn's disease and the remaining three with a final diagnosis of giardiasis and common variable immunodeficiency, primary lymphangiectasia and ileal carcinoid, respectively). Interestingly, in those patients with ileal Crohn's disease, US pattern differed completely from that observed in celiac patients based on a localised and marked bowel wall thickening. The details concerning the diagnostic accuracy of the six US signs evaluated are given in Table 3. In particular, the positive likelihood ratio (LR+) > 10 observed for increased GB volume, free abdominal fluid and enlarged mesenteric lymph nodes allowed a confirmatory strategy, whereas the negative likelihood ratio (LR-) of 0.1, observed for dilated small bowel loops and increased peristalsis, supported an exclusion strategy.

The k values ranging from 0.76 (for increased peristalsis) to 0.95 (for dilatation of small bowel loops) supported the good inter-observer agreement in the evaluation of the above reported US signs. Interestingly, strict gluten-free regimen obtained EmA negativization and a complete reversal of US abnormalities at one year.

In conclusion, the finding of increased GB volume, abdominal free fluid and mesenteric lymph nodes enlargement reliably and accurately predicts CD, whereas the lack of intestinal dilatation and increased peristalsis makes it possible to rule out the diagnosis. The specificity of US was maximized up to 99% in the presence of all the six US signs considered, with an obvious decrease in sensitivity (33%); moreover an LR + value of 50 allowed a confirmatory strategy accounting for a PPV of 80%. Conversely, in the presence of at least one US sign, sensitivity was 92% with a negative likelihood ratio value of 0.10 and a NPV of 99%, a diagnostic performance that was comparable to that of dilated small bowel loops.

Taking into account the above results, the actual role of US in the diagnostic strategy of celiac disease should be probably modulated according to the prevalence of the disease in the population under examination. As from Figure 1, the choice of the initial test depends on the estimated level of suspicion. In case of low CD prevalence (e.g. $\leq 10\%$), the negativity of the US signs, mainly the lack of "dilated small bowel loops with increased fluid content", can more effectively exclude the diagnosis and also can be very useful in discriminating functional from organic disease. Conversely, the concomitance of all the six US signs increases the probability from 10 to up 84%, still requiring a confirmatory intestinal biopsy. Facing with a moderate probability of CD (e.g. of about 30%) both

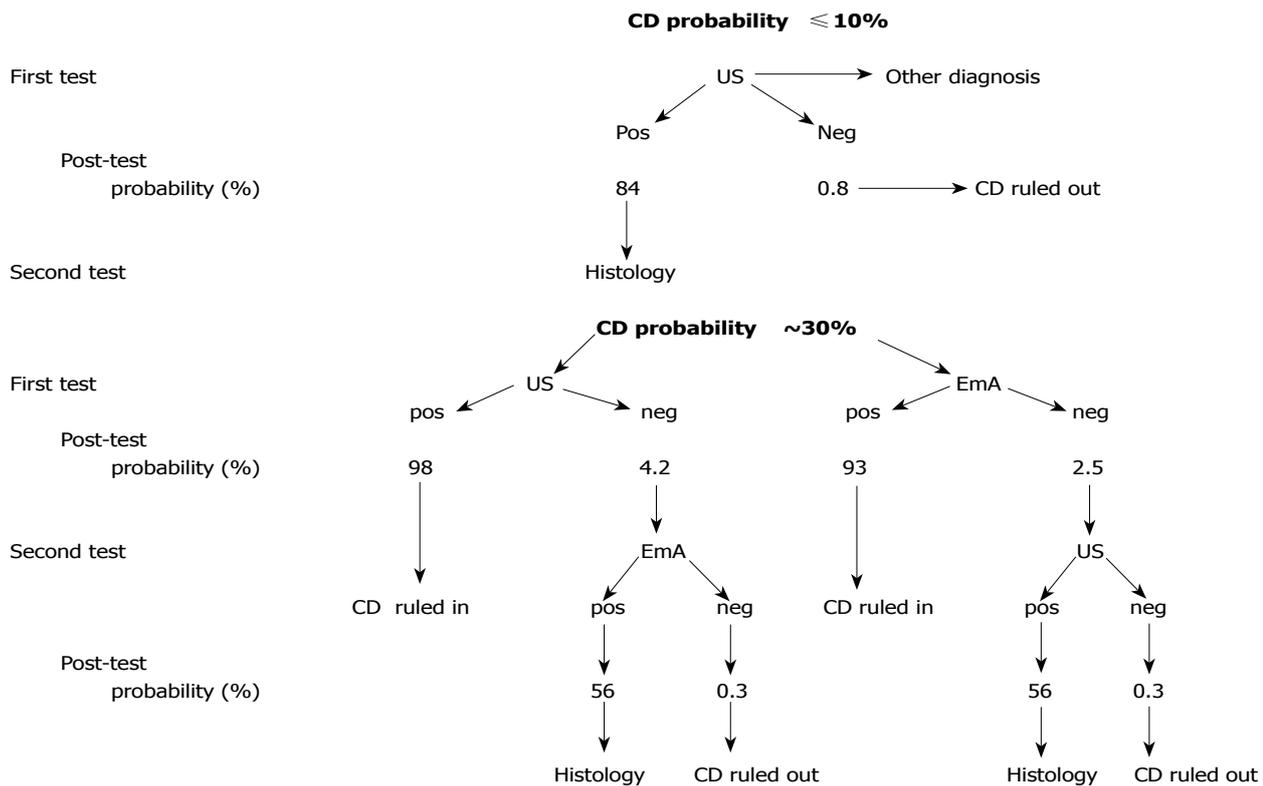


Figure 1 Diagnostic algorithm for celiac disease (CD), according to different pre-test probabilities of the disease. The post-test probability was calculated considering as a negative test the absence of “dilated small bowel loops with increased fluid content” (which showed the highest sensitivity: 92%, and the lowest LR- : 0.10) and as a positive test the presence of “all six US signs” (which has the highest specificity: 99%, and the highest LR+ : 50). The post-test probability for EmA was calculated considering a LR+ of 31 and LR- 0.06 (where LR indicate the likelihood ratio)²⁾.

Table 3 Diagnostic performance of six US parameters in predicting celiac disease (CD). Sensitivity, specificity, positive or negative likelihood ratio (LR+ and LR-), positive predictive value (PPV) and negative predictive value (NPV) (with corresponding 95% CI in parentheses).

| US parameters | Sens % | Spec % | LR +ve | LR - ve | PPV % | NPV % |
|---|-------------|------------|-----------------|-----------------|------------|-------------|
| Increased gallbladder volume (r.v. ≤ 20mL) | 73 (46-99) | 96 (92-99) | 17.0 (7.3-41.0) | 0.28 (0.1-0.7) | 57 (31-83) | 98 (95-100) |
| Dilated small bowel loops + increased fluid content (r.v. ≤ 2.5 cm) | 92 (76-100) | 77 (70-84) | 4.0 (2.8-5.5) | 0.10 (0.1-0.7) | 24 (11-36) | 99 (97-100) |
| Thickened small bowel wall (r.v. ≤ 3 mm) | 75 (50-99) | 91 (86-95) | 8.0 (4.4-14.5) | 0.27 (0.1-0.7) | 39 (19-59) | 98 (95-100) |
| Increased peristalsis | 83 (62-100) | 87 (82-92) | 6.6 (4.0-10.7) | 0.10 (0.05-0.7) | 34 (17-51) | 98 (96-100) |
| Free abdominal fluid | 50 (22-78) | 96 (93-99) | 12.5 (4.7-33) | 0.52 (0.3-0.9) | 50 (22-78) | 96 (92-99) |
| Enlarged mesenteric lymph nodes (r.v. ≤ 5 mm) | 42 (14-69) | 97 (95-99) | 15.6 (6.8-50.6) | 0.59 (0.4-0.9) | 55 (23-88) | 95 (92-99) |

IgA EmA testing and US can confirm the diagnosis, based on a PPV > 90%, a level at which the diagnostic role of histology could be challenged. Finally, independently of the test sequence, in the presence of both US and EmA results negativity, CD diagnosis can be confidently excluded as the post-test probability drops to less than 1% (NPV 99%). Obviously, in case of discordance, duodenal histology remains mandatory.

Overall, ultrasonography can accurately predict celiac disease but its place in the diagnostic algorithm should be varied according to the probability of the disease in a given population.

SPLANCHNIC CIRCULATION IN CELIAC DISEASE

Basal and postprandial splanchnic blood flow (i.e. that in superior mesenteric artery, SMA, and portal vein, PV) was assessed in celiacs by doppler-US. Uniformly, in fasting condition, there was an increase in SMA velocity and flow, with lower resistivity index, and a higher PV velocity and flow as compared to controls^[16-19]. As a consequence, when determined in postprandial phase all the above mentioned parameters showed a significantly lower variation as compared to that observed in healthy subjects^[17, 19]. Once

again, successful long term gluten-free diet reverted all the above abnormalities.

ABDOMINAL ULTRASOUND IN THE DIAGNOSIS OF THE COMPLICATION OF CELIAC DISEASE INTESTINAL LYMPHOMA AND SMALL BOWEL ADENOCARCINOMA

Enteropathy-associated T cell non-Hodgkin lymphoma (EATL-NHL), whose annual incidence is nearly 0.5-1 per million people, is actually confined to patients with a previously or concomitantly diagnosed CD, and also complicates the refractory form of the enteropathy. Moreover, an increased risk of small bowel adenocarcinoma, an exceedingly rare malignancy with an annual incidence rate of 0.6-0.7 per 100 000 of general population⁽⁶⁾, has also been reported.

In the presence of both these neoplasms, the typical US pattern is closely similar with evidence of the so called “bull’s eye”-, “target”- or “pseudokidney” sign related to an eccentric and localized bowel wall thickening⁽²⁰⁾.

In the above conditions, US is useful in both the disease location and staging; furthermore, it also allows both a detailed characterization of adjacent or distant lymph nodes and their sampling for histology, whenever indicated.

Overall, however, US should not be considered a first level technique in diagnosing superimposed intestinal lymphoma in CD patients, based on its low sensitivity even if performed by well trained operators. Accordingly, US as to be considered as ancillary to endoscopy in identifying and staging EATL in CD patients.

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Differences in oxidative stress dependence between gastric adenocarcinoma subtypes

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CONCLUSION: The iNOS-oxidative pathway may play an important role in TC, but moderately in PolyC and DC. DNA oxidation and protein nitration occur in the three subtypes. Based on the significant differences of NTYR levels, TC and PolyC appear as two distinct subtypes.

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Key words: Gastric neoplasms; Nitric oxide synthase; *H pylori*; Nitrotyrosine; 8-hydroxydeoxyguanosine; Oxidative stress

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Abstract

AIM: To investigate the extent of oxidative stress in pre-neoplastic and neoplastic gastric mucosa in relation to their pathological criteria and histological subtypes.

METHODS: A total of 104 gastric adenocarcinomas from 98 patients (88 infiltrative and 16 intraepithelial tumors) were assessed immunohistochemically for expression of iNOS and occurrence of nitrotyrosine (NTYR)-containing proteins and 8-hydroxy-2'-deoxyguanosine (8-OH-dG)-containing DNA, as markers of NO production and damages to protein and DNA.

RESULTS: Tumor cells staining for iNOS, NTYR and 8-OH-dG were detected in 41%, 62% and 50% of infiltrative carcinoma, respectively. The three markers were shown for the first time in intraepithelial carcinoma. The expression of iNOS was significantly more frequent in tubular carcinoma (TC) compared to diffuse carcinoma (DC) (54% vs 18%; $P=0.008$) or in polymorphous carcinoma (PolyC) (54% vs 21%; $P=0.04$). NTYR staining was obviously more often found in TC than that in PolyC (72% vs 30%; $P=0.03$). There was a tendency towards a higher rate of iNOS staining when distant metastasis (pM) was present. In infiltrative TC, the presence of oxidative stress markers was not significantly correlated with histological grade, density of inflammation, the depth of infiltration (pT), lymph nodes dissemination (pN) and pathological stages (pTNM).

INTRODUCTION

Gastric carcinoma is one of the most common neoplasms in the world, and most are adenocarcinomas. According to the Lauren classification^[5], two epidemiologically distinct types^[1-4] exist. Intestinal-type carcinoma (tubular carcinoma, TC) is associated with *H pylori* infection, chronic gastritis, atrophy, intestinal metaplasia, and dysplasia that evolve as a multi-step process into this type of cancer. Diffuse-type carcinoma (DC), which occurs more often in younger patients, is also associated with *H pylori* infection but not with atrophy and intestinal metaplasia, and is associated with a worse prognosis. Mixed polymorphous carcinoma (PolyC) encompasses tumors showing both glandular and diffuse components.

Inducible nitric oxide synthase (iNOS), which is synthesized *de novo* in response to mucosal inflammation in *H pylori*-associated gastritis^[6-10], produces large amounts of nitric oxide (NO) over long periods of time. Inflammatory cells also produce reactive oxygen species such as superoxide anion which is subsequently converted into hydrogen peroxide and hydroxyl radical^[11]. The reaction of NO with oxygen and superoxide produces nitrogen oxides and peroxynitrite, respectively. Overproduction of such reactive species leads to oxidation, nitration and nitrosation, resulting in protein, DNA and tissue damage. Nitrotyrosine (NTYR), formed by nitration of tyrosine

residues in proteins, can be used as a marker of nitrate damage *in vivo*^[12,13], and we^[6,14] and others^[8,9] have shown that its levels are elevated in *H pylori*-infected gastric mucosa. 8-Hydroxy-2'-deoxyguanosine (8-OH-dG) is one of the most commonly used markers of oxidative DNA damage^[15]. We^[6] and others^[16-18] have demonstrated increased levels of 8-OH-dG in *H pylori*-infected gastric mucosa. Oxidative stress has been suggested to play a major role in carcinogenesis^[19,20], but the mechanisms involved still remain unclear. Expression of iNOS in various carcinomas has been reported^[21-25], but the studies of stomach cancer were performed with small numbers of cases^[26-29] and they considered only limited pathological data^[30-34]. The presence of NTYR in some carcinomas was previously reported^[35-37], but no data, to our knowledge, on gastric cancer are available. Elevated levels of 8-OH-dG were demonstrated in some carcinomas^[35,36,38,39], but in only two studies on gastric carcinoma^[30,40].

In this study, we therefore aimed to investigate the extent of oxidative stress in pre-neoplastic and neoplastic gastric mucosa in relation to the cell compartment concerned and pathological criteria, especially the cancer subtype, and to identify subsets of cancers with a distinctive profile for the oxidative stress markers.

MATERIALS AND METHODS

Subjects

Our retrospective study included 98 consecutive French patients with gastric adenocarcinoma and 26 non-tumorous patients. All subjects were endoscopically biopsied for diagnostic purposes, between 1989 and 2000. Demographic information and medical history were obtained in all patients from the hospital records. The 104 carcinomas from the 98 patients were either infiltrative ($n=88$; 58 men and 30 women; mean age 70 years, range 30-98 years), or intraepithelial (*i.e.*, high-grade dysplasia; $n=16$), of which 6 had an adjacent infiltrative tumor (4 men and 2 women; mean age 64 years, range 54 to 75 years) and 10 had no coexisting infiltrative cancer (6 men and 4 women; mean age 70 years, range 44 to 85 years). Of the 88 infiltrative cancers, 57 were classified as tubular/intestinal (TC) (38 men and 19 women; mean age 64 years, range 44 to 76 years), 17 as diffuse (DC) (12 men and 5 women; mean age 68 years, range 49 to 92 years), and 14 as polymorphous (PolyC) (8 men and 6 women; mean age 70 years, range 57 - 98 years). *H pylori* was detected in 21% (19/92) of patients with carcinoma, including 10% (1/10) with intraepithelial carcinoma, 19% (11/54) with TC, 33% (4/12) with PolyC, and 19% (3/16) with DC.

Diagnostic biopsy samples with precursor lesions ($n=94$) were obtained from 57 of the 98 patients with gastric adenocarcinoma and from 26 randomly selected non-tumorous subjects. They were classified as low-grade dysplasia ($n=19$), of which 12 had no coexisting carcinoma (9 men and 3 women; mean age 68 years, range 43 to 88 years), and 7 were associated with invasive carcinoma, either adjacent ($n=3$) or distant ($n=4$). Of the 75 atrophic gastritis lesions, all but seven in association with intestinal metaplasia, 51 cases were in patients with intraepithelial or infiltrative cancer, either adjacent ($n=36$) or distant ($n=15$),

and 24 cases had no coexisting cancer. Among the latter 24 cases, 10 also showed low-grade dysplasia, and of the remaining 14, all but one had intestinal metaplasia (11 men and 3 women; mean age 64 years, range 24 to 90 years). *H pylori* infection was found in 32% (6/19) of patients with low-grade dysplasia and in 33% (25/75) of those with atrophic gastritis.

Diagnostic biopsy samples without precursor lesions ($n=48$) were obtained from non-tumorous mucosa of 38 of the 98 patients with gastric carcinoma and from 5 of the 12 non-tumorous patients with isolated low-grade dysplasia. The 43 biopsies from 38 gastric cancer patients were taken from mucosa surrounding ($n=36$) or distant from ($n=7$) the tumor. Mucosa was classified as normal ($n=8$, including 3 from non-cancerous subjects), or non-metaplastic gastritis ($n=40$, including 2 from non-cancerous subjects). *H pylori* infection was found in 23% (10/43) of subjects.

Histopathology and assessment of *H pylori* infection

We examined Bouin-fixed, paraffin-embedded archival biopsies from gastric mucosa. Sections (5- μ m thick) were stained using hematoxylin-eosin-saffron for histological diagnosis and with a modified Giemsa stain for identification of *H pylori*. Additional serial sections were used for immunohistochemistry. *H pylori* infection was graded as absent, mild, moderate or severe^[41]. In addition, a rapid urease test (CLO test) was performed in one third of cases.

The histological criteria were based on previous descriptions. According to the Lauren's classification^[5], infiltrative gastric cancers were classified as intestinal-type or tubular (TC), diffuse-type (DC), or mixed polymorphous (PolyC), the latter encompassing all tumors showing 5% or more glandular as well as 5% or more diffuse components^[42]. The DC consisted of dispersed cells or of minute cellular aggregates. Tumor grade was classified into three categories (well, moderately, or poorly differentiated). Dysplasia was categorized as low-grade or high-grade based on the severity of neoplastic features^[43,44]. High-grade dysplasia, as an intra-epithelial non-invasive neoplasia, was grouped with intraepithelial carcinoma according to the consensus terminology^[43,44].

Gastritis was classified and intestinal metaplasia (IM) was noted according to the guidelines of the updated Sydney system^[41]. In view of the small number of cases with severe gastritis ($n=4$), they were combined with the moderate gastritis cases ($n=36$) to form one group.

Pathologic stage (pTNM)

The pathologic stage (pTNM) was determined according to the newly revised classification of the American Joint Committee on Cancer (AJCC) and the International Union Against Cancer [UICC]^[45] for 53 patients who benefited from a curative resection either by gastrectomy ($n=42$), or endoscopy ($n=11$). Staging laparoscopy was performed for four subjects because of peritoneal metastasis. No surgery was performed for some cases because of either distant metastasis ($n=8$) or associated neoplasm ($n=5$). No staging data were available for 28 patients who underwent surgery in another centre.

We defined early gastric carcinoma as a tumor restricted to the mucosa or to the mucosa and submucosa (pT1) regardless of nodal status.

Immunohistochemistry

Bouin-fixed paraffin-embedded sections (4- μ m thick) were routinely deparaffinized in xylene and rehydrated through a series of ethanol solutions. The immunohistochemical procedures were performed as previously described^[6,7]. The avidin-biotin complex method was applied to the serial sections mounted on poly-L-lysine-coated plates. Briefly, after deparaffinization and inactivation of endogenous peroxidases, non-specific immunolabelling was blocked and overnight incubation was carried out with the primary antibody at 4°C. Primary antibodies used were as follows: (1) Polyclonal antibodies raised in the rabbit against iNOS (dilution 1:100), purified from rat livers with acute liver necrosis induced by iv administration of *Propionibacterium acnes* and lipopolysaccharide provided by one of us (HO)^[7,46,47]; (2) mouse monoclonal antinitrotyrosine antibody (dilution 1:250) supplied by Upstate Biotechnology (Lake Placid, NY); and (3) purified mouse monoclonal antibody against 8-hydroxy-2'-deoxyguanosine (N45.1, 3 μ g/mL)^[48,49]. A secondary biotinyl-labeled goat antirabbit or horse antimouse IgG serum and avidin-biotinylated horseradish peroxidase macromolecular complex (ABC Kit, Elite Vectastain, Vector Lab, Burlingame, CA) were sequentially used as previously described^[7]. Colorimetric reaction was performed with 3,3'-diaminobenzidine tetrachloride and counterstaining was carried out with hematoxylin. The degree of immunopositivity was expressed according to the percentage of positive cells as follows: <2%, negative (score 0); 2-10%, slightly positive (score 1); 10-50%, positive (score 2); and >50%, strongly positive (score 3). In addition, the intensity of staining was rated from weak to intense. Because the scoring system and the rating of intensities (data not shown) brought a great complexity that was useless for interpretation of the study, the results were simplified by collapsing in positive or negative groups. Sections in which the primary antibody was omitted or those treated with normal mouse serum instead of the primary antibody showed no staining. Furthermore, immunostaining of iNOS and NTYR was completely removed by absorption tests with iNOS purified from rat liver or with free NTYR (data not shown).

Statistical analysis

Statistical analysis was performed using the Fisher and Chi-square tests for contingency tables. $P < 0.05$ was considered statistically significant.

RESULTS

Correlations of the three markers with clinicopathological data and with pTNM pathologic classification and stage grouping are shown in Table 1, Table 2 and Table 3, respectively. The co-occurrence of staining for all the three markers is shown in Table 4.

iNOS immunostaining

Immunostaining for iNOS was located in the cytoplasm of

tumor cells (Figure 1A). It was more frequently observed in men than in women (Table 1), but the correlation was borderline ($P = 0.06$). Of the 85 infiltrative carcinoma cases, 41% showed weak to strong immunoreactivity. The staining was significantly correlated with infiltrative cancer histological subtype (Table 1), being markedly higher in TC than in DC ($P = 0.008$) and than in PolyC ($P = 0.04$). Comparing TC with DC + PolyC, the correlation remained significant ($P = 0.04$). However, in infiltrative TC, the iNOS immunoreactivity was not significantly correlated with either the depth of invasion (pT) or lymph node dissemination (pN) (Table 2). A higher rate of iNOS staining was observed when distant metastasis (pM) was present (Table 2), but this correlation did not reach statistical significance, presumably because of the small number of patients. The importance of iNOS in TC metastasis was reflected by its higher staining rate in stage IV than that in other stages (Table 3), although the difference was not statistically significant. The frequency of iNOS in intraepithelial carcinoma (not coexisting with infiltrative cancer - pTis) was lower than that in infiltrative TC (Table 1), but this was not statistically significant ($p = 0.10$). Similarly, staining was less common in early cancer than in other stages (Table 3), but not statistically significant ($p = 0.48$). The small number of DC and PolyC samples did not permit the study of any correlation of iNOS with pTNM and stage grouping criteria. There was no significant association of iNOS expression with histological grade for infiltrative TC [well ($n = 32$), moderately ($n = 3$) and poorly ($n = 9$) differentiated; iNOS positivity 56%, 46%, and 56%, respectively], *H pylori* infection (Table 1) or density of inflammation [weak ($n = 36$) vs strong ($n = 56$); iNOS positivity 42% and 38%]. In addition, iNOS expression was not found to depend on the cancer site (Table 1), such as body, cardia, gastric stump, and antrum with angulus and pylorus.

Non-metaplastic mucosa in the same patient, either adjacent to or distant from the tumor, showed lower epithelial reactivity than atrophic metaplastic gastritis mucosa from cancer and non-cancer patients (Table 1), but the tendency was not statistically significant ($P = 0.13$). A similar observation was found when comparing non-metaplastic mucosa and low-grade dysplasia.

Moreover, we detected iNOS expression in 44% (39/89) of infiltrating lymphocytes, regardless of their density and the carcinoma subtype.

NTYR immunostaining

NTYR immunostaining was also observed in the cytoplasm of tumor cells (Figure 1B). Of the 65 infiltrative carcinoma cases, 62% showed weak to strong immunoreactivity. The staining was significantly correlated with the histological subtype of infiltrative cancer, being significantly higher in TC than in PolyC ($P = 0.03$) (Table 1). However, when comparing TC with DC or PolyC + DC, the correlation was not statistically significant. In infiltrative TC, NTYR was not significantly correlated with the depth of invasion (pT), lymph node dissemination (pN) or distant metastasis (pM) (Table 2), nor was any relationship observed with pathological stages in TC (Table 3). The level of NTYR in intraepithelial carcinoma (not coexisting with infiltrative

Table 1 Correlation of staining with clinicopathologic data

| Clinicopathologic features ^a | iNOS ^b | | | NTYR ^b | | | 8OH-dG ^b | | |
|---|-------------------|------------------------------------|--|-------------------|------------------------------------|--|---------------------|------------------------------------|--|
| | No. of cases | | | No. of cases | | | No. of cases | | |
| | Total | positive (%) | | Total | positive (%) | | Total | positive (%) | |
| Infiltrative cancer subtypes | 85 | 35 (41) | | 65 | 40 (62) | | 82 | 41 (50) | |
| TC | 54 | 29 ¹ (54 ¹) | | 43 | 31 ² (72 ²) | | 54 | 28 (52) | |
| DC | 17 | 3 ¹ (18 ¹) | | 12 | 6 (50) | | 14 | 7 (50) | |
| PolyC | 14 | 3 ¹ (21 ¹) | | 10 | 3 ² (30 ²) | | 14 | 6 (43) | |
| Intraepithelial cancer | | | | | | | | | |
| Periinfiltrative cancer | 5 | 3 (60) | | 6 | 4 (67) | | 6 | 4 (67) | |
| Without infiltrative cancer | 7 | 1 (14) | | 7 | 6 (86) | | 10 | 5 (50) | |
| Topography of infiltrative cancer | | | | | | | | | |
| Cardia | 10 | 4 (40) | | 7 | 3 (43) | | 11 | 5 (45) | |
| Gastric stump | 12 | 6 (50) | | 9 | 5 (56) | | 8 | 3 (38) | |
| Antrum with angulus+pylorus | 45 | 18 (40) | | 35 | 22 (63) | | 41 | 22 (54) | |
| Body | 14 | 7 (50) | | 11 | 9 (82) | | 15 | 11 (73) | |
| Peritumorous mucosa | | | | | | | | | |
| Adjacent: N + no IM G | 15 | 5 (33) | | 23 | 9 (39) | | 30 | 10 (33) | |
| Adjacent: atrophy IM | 23 | 14 (61) | | 21 | 10 (48) | | 26 | 18 (69) | |
| At distance: N + no IM G | 3 | 1 (33) | | 6 | 1 (17) | | 5 | 2 (40) | |
| At distance: atrophy IM | 7 | 4 (57) | | 7 | 5 (71) | | 13 | 9 (69) | |
| Adjacent+distant: N + no IM G | 18 | 6 (33) | | 29 | 10 (34) | | 35 | 12 ³ (34 ³) | |
| Adjacent+distant: atrophy IM | 30 | 18 (60) | | 28 | 15 (54) | | 39 | 27 (69) | |
| Precancerous lesions | | | | | | | | | |
| Low grade dysplasia | 13 | 9 (69) | | 14 | 11 (79) | | 17 | 14 ³ (82 ³) | |
| Atrophy IM without cancer | 10 | 8 (80) | | 7 | 7 (100) | | 11 | 6 (55) | |
| Atrophy IM with + without cancer | 40 | 26 (65) | | 35 | 22 (63) | | 50 | 33 ³ (66 ³) | |
| <i>H pylori</i> infection | | | | | | | | | |
| Negative | 68 | 27 (40) | | 54 | 35 (65) | | 77 | 41 (53) | |
| Positive | 18 | 7 (39) | | 13 | 7 (54) | | 16 | 9 (56) | |
| Sex | | | | | | | | | |
| Male | 60 | 28 (47) | | 50 | 32 (64) | | 66 | 36 (55) | |
| Female | 30 | 8 (27) | | 29 | 20 (69) | | 35 | 18 (51) | |

(a) TC: Intestinal or tubular carcinoma; DC: Diffuse carcinoma; PolyC: Mixed polymorphous carcinoma; N: Normal; IM: Intestinal metaplasia; G: Gastritis. (b) iNOS: Inducible nitric oxide synthase; NTYR: Nitrotyrosine; 8OH-dG: 8-hydroxy-2'-deoxyguanosine. ¹TC vs DC $P = 0.008$; TC vs PolyC, $P = 0.04$; TC vs DC+PolyC, $P = 0.04$; ²TC vs PolyC, $P = 0.03$; ³non-metaplastic mucosa vs atrophic metaplastic mucosa low-grade dysplasia, $P = 0.005$.

cancer - pTis) was not significantly different from that in infiltrative TC (Table 1). Similarly, the level of NTYR in early gastric cancer was not different from that of other stages (Table 3). There was no significant association between NTYR expression and sex, cancer site (Table 1), histological grade in TC [well ($n = 28$), moderately ($n = 10$) and poorly ($n = 5$) differentiated; NTYR positivity 75%, 60%, and 80%, respectively], *H pylori* infection (Table 1) or density of inflammation [weak ($n = 27$) vs strong ($n = 45$); NTYR staining 63% and 64%].

Non-metaplastic mucosa in the same patient, either adjacent to or distant from the tumor, showed lower epithelial staining for NTYR than atrophic metaplastic mucosa from cancer and non-cancer patients (Table 1), but the tendency was not statistically significant ($P = 0.18$). A similar observation was found when comparing non-metaplastic mucosa and low-grade dysplasia (Figure 1F).

In addition, immunostaining of NTYR was detected in the cytosol of inflammatory cells at a higher rate in intraepithelial carcinoma ($n = 7$, 71%) than that in inflammatory cells in all subtypes of infiltrative cancer ($n = 67$, mean 33%).

8-OH-dG immunostaining

Staining was predominantly confined to the nuclei of tumor cells (Figures 1C and 1D). Because the lymphoid follicles were strongly reactive, they could be used as internal positive controls (Figure 1C). Of the 82 cases of infiltrative cancer, 50% showed strong immunoreactivity.

In contrast with iNOS and NTYR, no significant difference in the percentage of positive staining was observed among different histological subtypes of infiltrative cancer (Table 1). The presence of 8-OH-dG was not significantly associated with sex, cancer site (Table 1), histological grade in TC [well ($n = 32$), moderately ($n = 12$) and poorly ($n = 10$) differentiated; positivity 47%, 67%, and 50%, respectively], *H pylori* infection (Table 1), depth of invasion (pT), lymph node dissemination (pN), distant metastasis (pM) (Table 2), or pathological stage in TC (Table 3). The levels of 8-OH-dG in intraepithelial carcinoma (not coexisting with infiltrative cancer - pTis) (Figure 1E) and in infiltrative TC were not significantly different (Table 1). Immunostaining of 8-OH-dG in early cancer was similar to that in all other stages (Table 3).

Staining of 8-OH-dG in non-metaplastic mucosa from cancer patients was significantly lower than that in atrophic metaplastic mucosa from cancer and non-cancer patients and than that in low-grade dysplasia ($P = 0.005$) (Table 1). Immunoreactivity of 8-OH-dG was significantly higher in the nuclei of inflammatory cells in cases with mild inflammatory stroma (38%, 12/32) than that with dense inflammatory stroma (24%, 12/49).

Co-staining for iNOS, NTYR and 8-OH-dG

The expressions of all three markers were determined simultaneously in 62 cases (Table 4). The absence of all three markers was more frequent in PolyC (50%), but less frequent in TC (12%) and DC (16%). When iNOS was the

Table 2 Correlation of staining with pTNM pathological classification^a

| Pathological classification ^b | iNOS ^c | | | NTYR ^c | | | 8OH-dG ^c | | |
|--|--------------------|----|-------|--------------------|----|-------|---------------------|----|-------|
| | No. of cases | | | No. of cases | | | No. of cases | | |
| | Total positive (%) | | | Total positive (%) | | | Total positive (%) | | |
| pTis | 7 | 1 | (14) | 7 | 6 | (86) | 10 | 5 | (50) |
| pT1 | 18 | 7 | (39) | 16 | 12 | (75) | 16 | 10 | (63) |
| TC | 13 | 7 | (54) | 12 | 9 | (75) | 12 | 8 | (67) |
| DC | 3 | 0 | (0) | 2 | 2 | (100) | 2 | 2 | (100) |
| PolyC | 2 | 0 | (0) | 2 | 1 | (50) | 2 | 0 | (0) |
| pT2 | 7 | 3 | (43) | 8 | 6 | (75) | 8 | 4 | (50) |
| TC | 5 | 2 | (40) | 6 | 5 | (83) | 6 | 3 | (50) |
| DC | 0 | 0 | (0) | 0 | 0 | (0) | 0 | 0 | (0) |
| PolyC | 2 | 1 | (50) | 2 | 1 | (50) | 2 | 1 | (50) |
| pT3/T4 | 22 | 9 | (41) | 15 | 8 | (53) | 21 | 10 | (48) |
| TC | 12 | 7 | (58) | 8 | 6 | (75) | 12 | 6 | (50) |
| DC | 5 | 1 | (20) | 3 | 1 | (33) | 4 | 2 | (50) |
| PolyC | 5 | 1 | (20) | 4 | 1 | (25) | 5 | 2 | (40) |
| pN0 | 20 | 10 | (50) | 20 | 14 | (70) | 20 | 11 | (55) |
| TC | 16 | 8 | (50) | 16 | 13 | (81) | 16 | 10 | (63) |
| DC | 1 | 1 | (100) | 1 | 0 | (0) | 1 | 1 | (100) |
| PolyC | 3 | 1 | (33) | 3 | 1 | (33) | 3 | 0 | (0) |
| pN+ | 24 | 8 | (33) | 17 | 9 | (53) | 22 | 11 | (50) |
| TC | 10 | 6 | (60) | 7 | 4 | (57) | 10 | 4 | (40) |
| DC | 7 | 0 | (0) | 5 | 3 | (60) | 5 | 3 | (60) |
| PolyC | 7 | 2 | (29) | 5 | 2 | (40) | 7 | 4 | (57) |
| pM0 | 38 | 14 | (37) | 3 | 21 | (64) | 36 | 20 | (56) |
| TC | 25 | 12 | (48) | 2 | 17 | (77) | 25 | 15 | (60) |
| DC | 6 | 1 | (17) | 4 | 2 | (50) | 4 | 4 | (100) |
| PolyC | 7 | 1 | (14) | 7 | 2 | (29) | 7 | 1 | (14) |
| pM1 | 16 | 9 | (56) | 12 | 8 | (67) | 16 | 8 | (50) |
| TC | 9 | 7 | (78) | 8 | 6 | (75) | 9 | 5 | (56) |
| DC | 4 | 0 | (0) | 3 | 2 | (67) | 4 | 1 | (25) |
| PolyC | 3 | 2 | (67) | 1 | 0 | (0) | 3 | 2 | (67) |

(a) Determination according to the newly revised classification of the AJCC and UICC. (b) TC: Intestinal or tubular carcinoma; DC: Diffuse carcinoma; PolyC: Mixed polymorphous carcinoma. (c) iNOS: Inducible nitric oxide synthase; NTYR: Nitrotyrosine; 8OH-dG: 8-hydroxy-2'-deoxyguanosine

only positive marker found, its frequency was similar in the three infiltrative cancer subtypes (5-10%). When the only positive marker found was either NTYR or 8-OH-dG, its frequency was similar for both of them in the three cancer subtypes, but higher in DC (17%) compared to PolyC (10%) and TC (5-10%). The couple NTYR + 8-OH-dG was the only one found in PolyC and occurred at a similar frequency in the three cancer subtypes (20-25%). It was also the most common one in DC (25%) among the three possible couples. In TC, the two couples iNOS + NTYR and NTYR + 8-OH-dG occurred at the same frequency (20%). The couple iNOS + 8-OH-dG occurred rarely in TC and DC (8% and 2.5%). All three markers were simultaneously observed only in TC (25%).

DISCUSSION

The current study investigated the expression of iNOS, NTYR, and 8-OH-dG, as markers of NO production, nitrative damage of proteins, and oxidative DNA damage respectively, in gastric adenocarcinoma and pre-neoplastic mucosa. The expression of these markers was studied in relation to clinicopathological characteristics, including histological subtype, metastasis and pathological stages.

Table 3 Correlation of staining with stage grouping^a

| Stages ^b | iNOS ^c | | | NTYR ^c | | | 8OH-dG ^c | | |
|------------------------------------|--------------------|----|------|--------------------|----|-------|---------------------|----|-------|
| | No. of cases | | | No. of cases | | | No. of cases | | |
| | Total positive (%) | | | Total positive (%) | | | Total positive (%) | | |
| Stage 0 | 7 | 1 | (14) | 7 | 6 | (86) | 10 | 5 | (50) |
| Stage I | 22 | 9 | (41) | 21 | 15 | (71) | 21 | 12 | (57) |
| TC | 16 | 8 | (50) | 16 | 12 | (75) | 16 | 10 | (63) |
| DC | 3 | 0 | (0) | 2 | 2 | (100) | 2 | 2 | (100) |
| PolyC | 3 | 1 | (33) | 3 | 1 | (33) | 3 | 0 | (0) |
| Stage II+III | 13 | 5 | (38) | 10 | 4 | (40) | 12 | 5 | (42) |
| TC | 8 | 4 | (50) | 5 | 4 | (80) | 7 | 3 | (43) |
| DC | 2 | 1 | (50) | 2 | 0 | (0) | 2 | 2 | (100) |
| PolyC | 3 | 0 | (0) | 3 | 0 | (0) | 3 | 0 | (0) |
| Stage IV | 19 | 9 | (47) | 14 | 10 | (71) | 18 | 10 | (56) |
| TC | 9 | 7 | (78) | 8 | 6 | (75) | 9 | 5 | (56) |
| DC | 5 | 0 | (0) | 3 | 2 | (67) | 4 | 1 | (25) |
| PolyC | 5 | 2 | (40) | 3 | 2 | (67) | 5 | 4 | (80) |
| All stages (early cancer excluded) | 36 | 16 | (44) | 29 | 17 | (59) | 35 | 17 | (49) |
| TC | 20 | 12 | (60) | 17 | 13 | (76) | 20 | 10 | (50) |
| DC | 7 | 1 | (14) | 5 | 2 | (40) | 6 | 3 | (50) |
| DC+PolyC | 16 | 4 | (25) | 12 | 4 | (33) | 15 | 7 | (47) |
| Early cancer (pTis + pT1) | 25 | 8 | (32) | 23 | 18 | (78) | 26 | 15 | (58) |
| TC | 20 | 8 | (40) | 19 | 15 | (79) | 22 | 13 | (59) |
| DC | 3 | 0 | (0) | 2 | 2 | (100) | 2 | 2 | (100) |
| DC+PolyC | 5 | 0 | (0) | 4 | 3 | (75) | 4 | 2 | (50) |

(a) Determination according to the newly revised classification of the AJCC and UICC. (b) TC: Intestinal or tubular carcinoma; DC: Diffuse carcinoma; PolyC: Mixed polymorphous carcinoma. (c) iNOS: Inducible nitric oxide synthase; NTYR: Nitrotyrosine; 8OH-dG: 8-hydroxy-2'-deoxyguanosine.

We found, for the first time, a positive correlation of gastric cancer subtype with both iNOS expression and NTYR staining, but not with 8-OH-dG staining. Indeed, the prevalence of iNOS staining in TC (54%) greatly exceeded that in DC (18%) and PolyC (21%), suggesting that iNOS probably plays an important role in TC, but is weakly involved, if at all, in DC and PolyC subtypes. We also found for the first time, nitrated proteins present in all cancer subtypes. Similarly to iNOS expression, the prevalence of NTYR staining in TC (72%) greatly exceeded that in PolyC (30%), but the difference was not significantly different from that in DC (50%). Half of the infiltrative carcinoma cases showed 8-OH-dG staining in cancerous cells, regardless of the subtypes.

The prevalence of iNOS staining we found in gastric infiltrative carcinoma (41%) was very similar to that reported by Feng *et al.*^[32], but was slightly lower than that reported in two other studies^[26,31] that used immunohistochemistry as we did. However, two studies from the same laboratory reported different results. Using RT-PCR, Son *et al* found expression of iNOS in 100%^[27] and 65%^[34] of gastric cancer. Rajnakova *et al*^[50] were not able to detect iNOS staining in 80% of gastric adenocarcinomas (in their first paper), whereas the same authors found it in all cases in a more recent study^[33]. Chang *et al*^[30] reported higher iNOS expression in cancerous tissue than that in adjacent non-cancerous mucosa, for which the pathological status was not reported. Concerning iNOS, histological subtype was not considered in four studies^[27,29,30,34]. Two studies^[26,33] did not

Table 4 Co-occurrence of immunostaining for iNOS, NTYR and 8-OH-dG^a

| Markers | No. of samples (%) | | |
|----------------------|---------------------------|---------------------------|------------------------------|
| | TC ^b n = 40 | DC ^b n = 12 | PolyC ^b n = 10 |
| All negative | 5 (12) | 2 (16) | 5 (50) |
| One positive | | | |
| iNOS | 2 (5) | 1 (8) | 1 (10) |
| NTYR | 4 (10) | 2 (17) | 1 (10) |
| 8-OHdG | 2 (5) | 2 (17) | 1 (10) |
| Two positive | | | |
| iNOS + NTYR | 8 (20) | 1 (8) | 0 (0) |
| NTYR + 8-OHdG | 8 (20) | 3 (25) | 2 (20) |
| iNOS + 8-OHdG | 1 (2.5) | 1 (8) | 0 (0) |
| All positive | | | |
| iNOS + NTYR + 8-OHdG | 10 (25) | 0 (0) | 0 (0) |

(a) iNOS: Inducible nitric oxide synthase; NTYR: Nitrotyrosine; 8OH-dG: 8-hydroxy-2'-deoxyguanosine. (b) TC: Intestinal or tubular carcinoma; DC: Diffuse carcinoma; PolyC: Mixed polymorphous carcinoma.

report a difference in relation to Lauren's classification, but one of them included only 19 cases (53% positive)^[26] and all cases were stained in the other one^[33]. We^[6] and others^[8,9] have previously shown the occurrence of NTYR-containing proteins in *H pylori*-associated gastritis preceding TC, but no data are available on gastric cancer. The marker of oxidative DNA damage, 8-OH-dG, has been reported in only two studies^[30,40]. Our findings can not be compared with these published data because of a difference in methodologies. Lee *et al*^[40] and Chang *et al*^[30] found 8-OH-dG in DNA extracted from cancer tissue using HPLC-electrochemical detection. This technique does not allow localization of the cellular compartment concerned (tumor cells and/or inflammatory cells), unlike our immunohistochemical method. This is particularly important because of the strong reactivity of 8-OH-dG in lymphoid follicles. Our results also supported the previous observations concerning the absence of an association of iNOS with the degree of differentiation^[31-33,50]. In addition, we found an absence of relationship of NTYR and 8-OH-dG with the degree of differentiation.

In contrast with previous reports which studied relationships with respect to only some pTNM categories (*e.g.*, pM often missing), we investigated the presence of the three markers as a function of all components of pTNM and complete staging. We found a tendency towards higher levels of iNOS in TC when distant metastasis (pM) was present, suggesting that high levels of iNOS oxidative stress may stimulate tumor progression^[51]. However, the levels of NTYR and 8-OH-dG were not related to the presence of distant metastasis. In agreement with previous findings^[31], we found no correlation between iNOS staining and depth of invasion (pT). One study^[32] showed increased staining of iNOS with depth of invasion, but without statistical significance. Similarly, in our study, the levels of NTYR and 8-OH-dG were not related to the depth of invasion. To our best of knowledge, this is the first study to investigate the three markers in gastric intraepithelial (pTis) and early gastric cancers. There is a tendency for a lower level of iNOS in

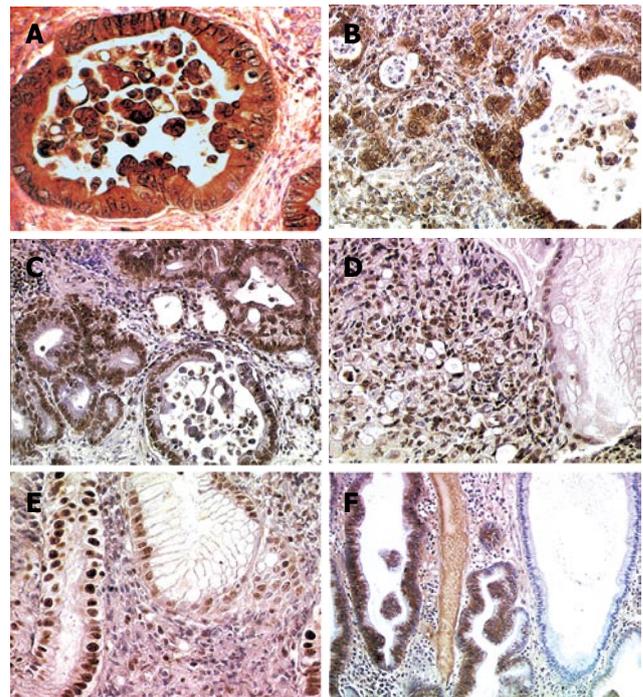


Figure 1 Immunohistochemical staining in human gastric adenocarcinoma and precancerous mucosa. **A:** Intense cytoplasmic immunostaining of iNOS in tumor cells of tubular infiltrative carcinoma (TC), well differentiated; **B:** Intense cytoplasmic immunostaining of NTYR in tumor cells of TC, moderately differentiated; **C:** Intense nuclear immunostaining of 8-OH-dG in tumor cells of TC, well differentiated, note the reactivity of the lymphoid follicle (upper left); **D:** Intense nuclear immunostaining of 8-OH-dG in tumor cells of diffuse carcinoma, note the weak reactivity of some cells of a normal crypt (right); **E:** Intense nuclear immunostaining of 8-OH-dG in high-grade dysplastic epithelium, note the weak reactivity of a normal crypt (right); and **F:** Intense cytoplasmic immunostaining of NTYR in low-grade dysplastic epithelium, note the absence of reactivity of a normal crypt (right).

intraepithelial carcinomas and early gastric cancer than that in infiltrative carcinomas, but further studies with larger number of patients would be required to judge the statistical significance. In contrast to a previous report^[32], we neither found any relationship of iNOS with lymph node dissemination (pN), nor any relationship of NTYR or 8-OH-dG with pN.

The levels of the three markers did not vary significantly according to cancer site (especially gastric stump, at high risk for cancer), or with the density of inflammation or *H pylori* infection. During the sequential steps from gastritis to TC, we^[6,7] and others^[8,16] have shown that iNOS produced continuously by *H pylori*-induced inflammatory cells plays a crucial role in the initiation of the carcinogenic process. In cancer, the absence of any relationship between iNOS and the two other markers with inflammation suggests that inflammation-induced damage does not contribute markedly to the final stages of the neoplastic disease. Only iNOS was more often expressed in males as compared with females, consistent with the higher frequency of gastric cancer in males as compared with females, but this reached only borderline significance.

Sequential steps (atrophic gastritis, intestinal metaplasia, and dysplasia) of precancerous changes precede the intestinal-type of gastric carcinoma^[2]. We found an increase

in epithelial levels of iNOS, NTYR, and 8-OH-dG from non-metaplastic non-atrophic gastritis to atrophic metaplastic gastritis and low-grade dysplasia, but reached statistical significance only for 8-OH-dG, thus supporting our previous results^[6]. Therefore, epithelial over-expression of these three markers is an early event in the neoplastic process leading to the intestinal cancer which occurs at the precancerous stage, in which certain genetic modifications are also observed frequently^[11].

Our study suggests that the iNOS-oxidative pathway plays a minimal role in PolyC, as none of the three markers measured were found in half of the patients, and NTYR was detected less frequently in PolyC compared to the other cancer subtypes. In DC, one or two markers were present in 83% of subjects, suggesting a higher level of oxidative processes, but DC was not dependent mainly on the iNOS-oxidative pathway. In contrast, in TC, the strong oxidative process mediated through iNOS may be important in gastric carcinogenesis, as only 12% of subjects had none of the three markers measured, and 25% of subjects showed all these markers simultaneously. In addition, our study suggests that iNOS-associated oxidative stress is an early event in intestinal-type carcinogenesis. These findings support the traditional identification of TC and DC as two distinct tumor entities. Our study is also in accordance with the identification of PolyC as a distinct histotype of gastric carcinoma.

In conclusion, our study shows (1) the occurrence of iNOS, NTYR and 8-OH-dG in tumor cells of infiltrative gastric adenocarcinoma and epithelial cells of precancerous mucosa, as well as in intraepithelial carcinoma, (2) a correlation of both iNOS and NTYR with cancer subtype, (3) no association of the three markers with the degree of inflammation or with *Hi pylori* infection, and (4) that oxidative stress occurs in the three cancer subtypes, and the iNOS-oxidative pathway may be important in gastric carcinogenesis in TC, but its involvement is minimal in PolyC and moderate in DC.

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Significance of Survivin and PTEN expression in full lymph node-examined gastric cancer

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Abstract

AIM: To study the relationship between Survivin and PTEN expression and lymph node metastasis, depth of invasion and prognosis of gastric cancer patients in China.

METHODS: Specimens of gastric cancer tissue were collected from the Affiliated Hospital of Jiangnan University. All the 140 patients had complete examination data. All lymph nodes were found by the fat-clearing method. The interrupted serial 4-micron sections, routine hematoxylin and eosin staining and immunohistochemical methods were used to detect the lymph node metastases. Gastric cancer tissue microarray was performed to test the expression of Survivin and PTEN (17A) in gastric cancer by immunohistochemical method. All data were processed using χ^2 test, Fisher's exact test, Kaplan-Meier Log-rank method and Cox multivariate analysis (SPSS 12.0 software).

RESULTS: One hundred and eighteen specimens were used in our tissue microarray (utilization rate was 82.4%). A total of 7580 lymph nodes were found. Metastases were found in 90 specimens and 1618 lymph nodes were detected. The positive rate of Survivin and PTEN expression was 52.5% (62/118) and 76.2% (90/118), respectively. A highly positive correlation was found between Survivin and PTEN expression ($\chi^2=4.17$, $P=0.04$). Survivin expression was positively correlated with UICC N stage ($\chi^2=8.69$, $P=0.03$) and histological classifica-

tion ($\chi^2=4.41$, $P=0.04$) by χ^2 tests. PTEN expression was positively correlated with depth of invasion ($P=0.02$) and histological classification ($\chi^2=5.47$, $P=0.02$). But Survivin and PTEN expressions were not related with prognosis of gastric cancer patients. A significant correlation between lymph node metastasis and prognosis was demonstrated by Cox multivariate analysis ($\chi^2=4.85$, $P=0.028$).

CONCLUSION: Survivin is positively correlated with PTEN expression in gastric cancer and is a molecular marker of lymph node metastasis while PTEN expression is a molecular marker of advanced gastric cancer. UICC N stage is the most important prognostic factor of gastric cancer in China.

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Key words: Gastric cancer; Survivin; PTEN; Tissue microarray

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INTRODUCTION

Gastric carcinoma is one of the commonest malignancies and the first killer among all tumors in China^[1]. When the disease is diagnosed, it has been in its advanced stage with lymph node metastasis. Looking for molecular markers to predict the depth of invasion and lymph node metastasis of gastric cancer has important significance.

Repression of apoptosis has been observed in gastric cancer^[2]. Survivin, a new member of IAP family, is a powerful apoptosis repression factor. It has been implicated in the control of cell cycle kinetics and cell proliferation^[3]. Survivin can be found during embryonic and fetal development but is completely down-regulated and undetectable in normal adult tissues and is prominently expressed in many human malignancies^[4]. PTEN/MMAC1/TEP1, a novel tumor suppressor gene, is located on chromosome band 10q 23.3. This gene has three different names: *PTEN* (phosphatase and tensin homologue deleted on chromosome ten)^[5], *MMAC1* (mutated in multiple advanced cancer 1)^[6] and *TEP1* (TGF- β -regulated and

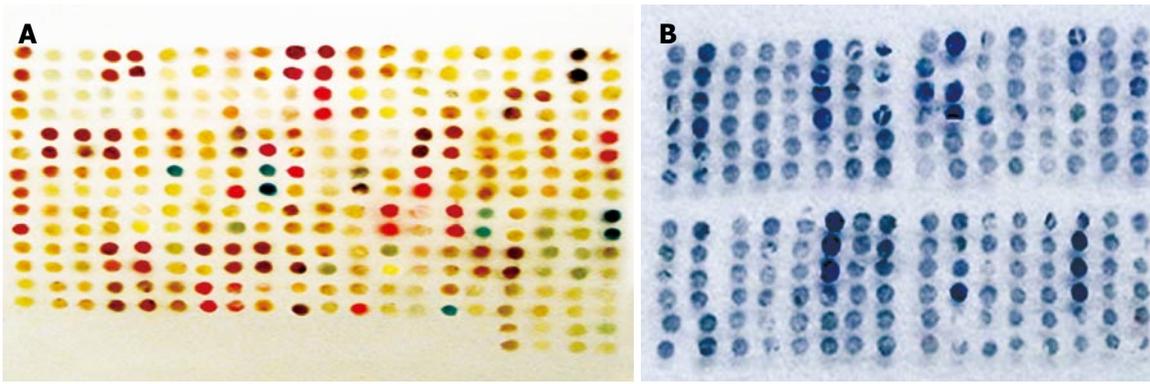


Figure 1 Paraffin block for gastric cancer microarray with 288 tissue cylinders (A) slide for gastric cancer microarray with 420 tissue cylinders (B)

epithelial cell-enriched phosphatase 1)^[7], and codes for the 403 amino acid protein with tyrosine phosphatase activity. It contains a region of homology to tensin and auxillin, cytoskeletal proteins that interact with adhesion molecules. PTEN encoding products could dephosphorate PIP3 that could inhibit cell growth and increase cell apoptosis. It also could inhibit cell invasion and metastasis by dephosphorating focal adhesion kinase (FAK) and restrain cell differentiation by inhibiting mitogen-activated protein kinase (MAPK) signal pathway^[8-10].

It is still controversial about the correlation between Survivin and PTEN expression and depth of invasion and lymph node metastasis as well as the prognosis significance of Survivin and PTEN expression in gastric cancer. In this study, we used tissue microarray and immunohistochemical technologies to study the relationship between Survivin and PTEN expression in gastric cancer and their relation with the depth of invasion, lymph node metastasis of gastric cancer and the prognosis of gastric cancer patients.

MATERIALS AND METHODS

Materials

Specimens of gastric cancer tissue were collected from the Affiliated Hospital of Jiangnan University. All the 140 patients had complete examination data (99 males and 41 females, median age 58 years, ranging 26 - 77 years). No patients received radiotherapy and chemotherapy before surgery. Depth of invasion and lymph node metastasis were staged by the standards of UICC. No patients were diagnosed as having gastric adenocarcinoma which was classified into well-differentiated and poorly-differentiated adenocarcinoma. Size was calculated according to the biggest diameter of tumor.

Pathological examination

All the 140 specimens of gastric cancer tissue were taken at the distance of 0.5-1 cm. Routine pathological examination was performed to determine the depth of invasion and histological classification of gastric cancer. All lymph nodes were found by the fat-clearing fat method (>15/case)^[11]. The interrupted 4-micron sections, routine hematoxylin and eosin staining and immunohistochemical SP methods (CK18 + EMA, Vector Laboratory Co.) were used

to detect the lymph node metastasis.

Tissue microarray

We took core needle biopsies with a diameter of 0.6 mm from donor paraffin-embedded cancer tissue blocks (2-4 dots per specimen) using a dedicated tissue array instrument (Beecher Instruments, New Jersey, USA). Slides for tissue microarray were read as previously described^[12]. Standard dots which could not be analyzed, included dots having no tumor tissue, the area of deflection being higher than 50% of dots, the area of tumor being less than 10% of dots, location of dots being unclear.

Immunohistochemistry

The primary polyclonal antibody to Survivin (RAB-0536, NeoMarkers; ready-to-use), monoclonal antibody to PTEN (clone 17A, NeoMarkers; ready-to-use) and SP kit were purchased from Fujian Maxin Ltd (China). Immunohistochemical staining of Survivin and PTEN was performed. Diaminobenzidin (DAB) was used for color development. The positive result of Survivin and PTEN showed brown color of cytoplasm.

Statistical analysis

Statistical software package SPSS 12.0 was used. Depth of invasion and lymph node metastasis associated with Survivin expression were analyzed by χ^2 test or Fisher's exact test. Survival analysis was conducted by the Kaplan-Meier method and survival characteristics were compared using the log rank test. The Cox proportional hazard regression model was used to compare the relative influences of different prognostic factors. $P < 0.05$ was considered statistically significant.

RESULTS

Tissue microarray

All the 140 specimens were embedded for two tissue microarrays. One included 288 cores, the other included 420 cores. According to the standard dots that could not be analyzed, 118 specimens could be analyzed in our study (Figure 1). The utilization rate of tissue microarray was 84.3%.

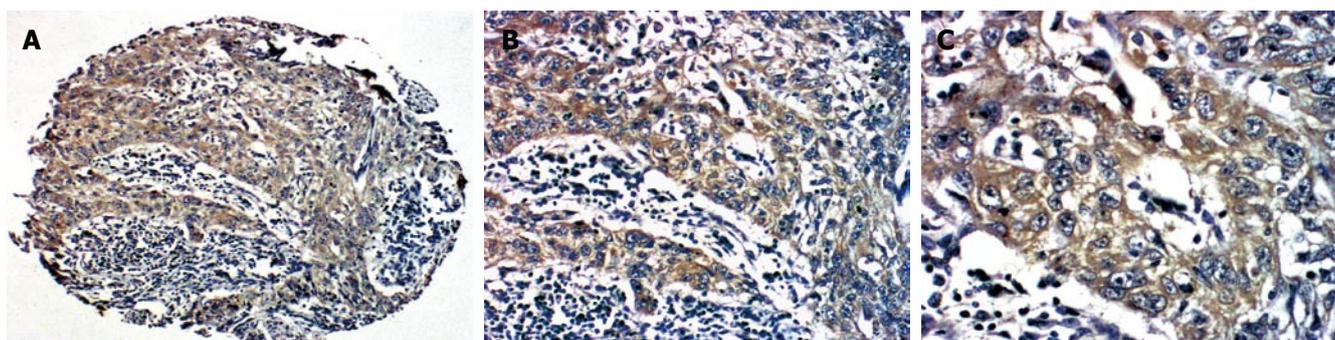


Figure 2 Immunohistochemical results of Survivin expression (A: $\times 100$; B: $\times 200$; C $\times 400$)

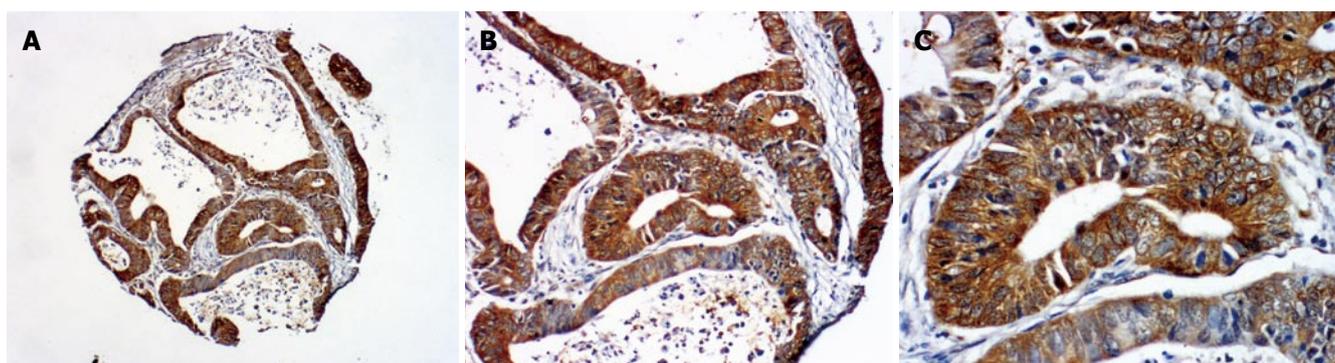


Figure 3 Immunohistochemical results of PTEN expression (A: $\times 100$ B: $\times 200$ C: $\times 400$)

Table 1 Correlation between Survivin and PTEN expression and pathological features

| | Survivin expression (n = 118) | | P | PTEN expression (n = 118) | | P |
|-----------------------|----------------------------------|----|------|------------------------------|----|------|
| | + | - | | + | - | |
| Gender | | | | | | |
| Male | 46 | 36 | | 59 | 23 | |
| Female | 16 | 20 | | 31 | 5 | |
| Age(yr) | | | | | | |
| <60 | 33 | 31 | | 44 | 20 | |
| ≥ 60 | 29 | 25 | | 46 | 8 | |
| Size | | | | | | |
| D<5cm | 26 | 25 | | 41 | 10 | |
| D ≥ 5 cm | 36 | 31 | | 49 | 18 | |
| Depth of invasion | | | | | | 0.02 |
| T ₁ | 4 | 7 | | 5 | 6 | |
| T ₂ | 12 | 6 | | 14 | 4 | |
| T ₃ | 19 | 19 | | 34 | 4 | |
| T ₄ | 27 | 24 | | 37 | 14 | |
| Histological type | | | 0.04 | | | 0.02 |
| Well-differentiated | 28 | 15 | | 38 | 5 | |
| Poorly-differentiated | 34 | 41 | | 52 | 23 | |
| Lymph node metastasis | | | 0.03 | | | |
| N ₀ | 18 | 10 | | 23 | 5 | |
| N ₁ | 18 | 9 | | 19 | 8 | |
| N ₂ | 14 | 15 | | 21 | 8 | |
| N ₃ | 12 | 22 | | 27 | 7 | |

Pathological features

All the 118 patients had complete examination data (82 males and 36 females, median age 58 years, ranging 26 - 77

years). The median size of tumor was 5.4 cm (ranging 0.8 - 15 cm). The depth of invasion was T₁ in 11, T₂ in 18, T₃ in 38, and T₄ in 51 patients, respectively. A total of 7580 lymph nodes were found in 118 cases (64.2/case, ranging 17 - 157 nodes, median number being 62 nodes) and 1618 lymph node metastases were found in 90 cases (UICC N stage, N₀ in 28, N₁ in 27, N₃ in 29, and N₄ in 34 cases, respectively) by the fat-clearing method. Well-differentiated gastric adenocarcinoma was found in 43 cases and poorly-differentiated gastric adenocarcinoma was found in 85 cases.

Correlation between expressions of Survivin and PTEN

Among the 118 patients, accumulation of Survivin in cytoplasm was found in 62 (52.5%, 62/118) cases and PTEN was detected in 90 cases (76.3%, 90/118) (Figure 2). There was a positive correlation between expressions of Survivin and PTEN ($\chi^2 = 4.17$, $P = 0.04$) (Figure 3).

Correlation between Survivin expression and pathological features

The positive rate of Survivin expression in UICC N stage was 65.5% (18/28) for N₀, 67.9% (18/27) for N₁, 48.3% (14/29) for N₂, 36.1% (12/34) for N₃, respectively. There was a significant correlation between Survivin and N stages of UICC ($\chi^2 = 8.69$, $P = 0.03$) by χ^2 tests. The positive rate of Survivin expression was 67.5% (28/43) in well-differentiated adenocarcinoma and 46.2% (34/75) in poorly-differentiated adenocarcinoma. There was a significant correlation between Survivin expression and histological classification ($\chi^2 = 4.41$, $P = 0.04$) by χ^2 test. No correlation

was found between Survivin expression and other pathological features (Table 1).

Correlation between PTEN expression and pathological features

The positive rate of PTEN expression was 45.5% (5/11) for T1, 77.8% (14/18) for T2, 89.5% (34/38) for T3, 72.6% (37/51) for T4, respectively. A highly positive correlation was found between depth of invasion and PTEN expression ($P=0.02$) by Fisher's exact test. The positive rate of PTEN expression was 88.4% (38/43) in well-differentiated adenocarcinoma and 69.3% (52/75) in poorly-differentiated adenocarcinoma. There was a significant correlation between PTEN expression and histological classification ($\chi^2=5.47$, $P=0.02$) by χ^2 test. No correlation was found between PTEN expression and other pathological features (Table 1).

Survival characteristics

Univariate analysis showed that depth of invasion ($\chi^2=12.25$, $P<0.0001$) and UICC N stage ($\chi^2=20.23$, $P<0.0001$) were significantly related with survival time. Multivariate analysis of these predictive variables revealed that only the UICC N stage ($\chi^2=4.85$, $P=0.028$) was not related with survival time.

The expression of Survivin and PTEN was not related with survival time. But when the survival time was longer than 7 mo, Survivin positive group had a worse prognosis than Survivin positive group ($P=0.047$).

DISCUSSION

Survivin, a structurally unique member of IAP family, is expressed in mitosis in a cell cycle-dependent fashion and localized in components of the mitotic apparatus^[3]. It is potentially involved both in inhibition of apoptosis and in control of cell division^[13,14]. Survivin is found in most human cancers but undetectable at a very low level in differentiated adult tissues^[4]. In most cancers, expression of Survivin is correlated with reduced apoptotic index, poor prognosis, and increased risk of recurrence^[15-18]. The relation between Survivin expression, depth of invasion and lymph node metastasis in gastric cancer is not well known. Miyachi *et al*^[19] found that Survivin expression is related with lymph node metastasis but not with depth of invasion. Tsuburaya *et al*^[20] showed that Survivin mRNA expression is related with depth of invasion but not with lymph node metastasis. The concordance of mRNA and protein is controversial. Our results were based on tissue microarray, immunohistochemistry, complete pathological data and complete lymph node examination data. We found that Survivin expression was higher in well-differentiated gastric adenocarcinoma than in poorly-differentiated gastric adenocarcinoma and was correlated with lymph node metastasis. The prognosis significance of Survivin expression is not well known. Wang *et al*^[21] found that Survivin positive expression in nuclei is correlated survival time, but its positive expression in cytoplasm is not correlated with survival time. We did not find any relation between Survivin expression and survival time.

But when the survival time was longer than 7 mo, Survivin positive group had a worse prognosis than PTEN positive group.

PTEN, a tumor suppressor gene, is located on chromosome band 10q 23.3. PTEN encoding products could inhibit cell growth and increase cell apoptosis by dephosphorylating PIP3. It also could inhibit cell invasion and metastasis by dephosphorylating focal adhesion kinase (FAK) and restrain cell differentiation by inhibiting mitogen-activated protein kinase (MAPK) signal pathway^[8-10]. The relation between PTEN expression, depth of invasion and lymph node metastasis is still unknown. Zheng *et al*^[22] found that PTEN expression is related with lymph node metastasis and depth of invasion. Dong *et al*^[22] found that PTEN expression is related with lymph node metastasis but not with depth of invasion. Our results showed that PTEN expression was higher in advanced gastric cancer than in early gastric cancer and was not related with lymph node metastasis. The prognosis significance of PTEN expression in gastric cancer is still unknown. Li *et al*^[23] found that PTEN expression is not related with survival time. We did not find any correlation between PTEN expression and survival time using tissue microarray and PTEN monoclonal antibody (17A)

Survivin and PTEN can regulate cell cycle and apoptosis, but they have adverse biological effects. Dong *et al*^[22] showed that PTEN expression is negatively related with Survivin expression. However, we found a positive relation between Survivin and PTEN expression.

Lymph node metastasis is the most important marker of prognosis of gastric cancer patients^[4,14]. Our study has confirmed that lymph node stage of gastric cancer is an independent prognosis marker of gastric cancer.

In conclusion, Survivin expression is positively related with PTEN expression in gastric cancer. They are not related with prognosis of gastric cancer. Survivin and PTEN expression is a molecular marker of advanced gastric cancer but not a molecular marker of lymph node metastasis in gastric cancer. UICC N stage is the most important prognostic factor in gastric cancer.

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LIVER CANCER

Effects of *Terminalia arjuna* bark extract on apoptosis of human hepatoma cell line HepG2

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Abstract

AIM: To investigate the effects of *Terminalia arjuna* (*T. arjuna*) extract on human hepatoma cell line (HepG2) and its possible role in induction of apoptosis.

METHODS: Human hepatoma cells were treated with different concentrations of ethanolic extract of *T. arjuna* and its cytotoxicity effect was measured by trypan blue exclusion method and lactate dehydrogenase leakage assay. Apoptosis was analyzed by light and fluorescence microscopic methods, and DNA fragmentation. The mechanism of apoptosis was studied with expression of p53 and caspase-3 proteins. Glutathione (GSH) content was also measured in HepG2 cells after *T. arjuna* treatment.

RESULTS: *T. arjuna* inhibited the proliferation of HepG2 cells in a concentration-dependent manner. Apoptotic morphology was observed in HepG2 cells treated with *T. arjuna* at the concentrations of 60 and 100 mg/L. DNA fragmentation, accumulation of p53 and cleavage of procaspase-3 protein were observed in HepG2 cells after the treatment with *T. arjuna*. The depletion of GSH was observed in HepG2 cells treated with *T. arjuna*.

CONCLUSION: *T. arjuna* induced cytotoxicity in HepG2 cells *in vitro*. Apoptosis of HepG2 cells may be due to the DNA damage and expression of apoptotic proteins. Depletion of GSH may be involved in the induction of apoptosis of HepG2 cells.

Keywords: Human hepatoma cell line; *Terminalia arjuna*; Apoptosis; p53; Caspase-3; GSH

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<http://www.wjgnet.com/1007-9327/12/1018.asp>

INTRODUCTION

Human hepatocellular carcinoma (HCC) constitutes about 85% of primary liver cancers recorded in cancer data banks. Globally, around 440 000 new cases of HCC occur annually, accounting for around 5.5% of all human cancer incidences^[1]. The synchronous occurrence of HCC may be due to different risk factors, such as chronic viral hepatitis B or hepatitis C infection, aflatoxin exposure, alcohol consumption and iron overload^[2]. Even though curative therapy can be achieved by surgical treatments such as resection or liver transplantation, treatment of unresectable HCC is very disappointing because many tumors stay asymptomatic for a long time and in the absence of effective screening programs^[3]. Furthermore, chemotherapeutic agents such as doxorubicin or its derivative epirubicin and cisplatin, have exhibited response rates up to 15-20% after systemic administration^[4]. Interestingly, a number of non-nutrient chemicals from plants and fruits have also been reported to possess anticancer activity. Certain products from plants are known to induce apoptosis in neoplastic cells but not in normal cells^[5].

Terminalia arjuna (Roxberg), locally known as kumbuk belonging to the family of *Combretaceae*, has a long history of medicinal uses in India including cancer treatment. Besides, HepG2 cell line represents one of the most widely used experimental model for *in vitro* studies on HCC^[6]. Therefore, it is of interest to investigate the effects of ethanolic extract of *T. arjuna* bark on human hepatoma cell line (HepG2).

MATERIALS AND METHODS

Chemicals and antibodies

RPMI-1640 and sodium pyruvate were purchased from Biochrom, Berlin, Germany. Penicillin, streptomycin and fetal bovine serum (FBS) were purchased from Gibco, Germany. Primary antibodies such as anti-p53 and anticaspase-

pase-3 were purchased from Novocastra Laboratories Ltd, Newcastle, UK and Transduction Laboratories, Lexington, UK, respectively.

Drug preparation

T. arjuna bark was coarsely powdered and soaked in 95% ethanol and kept for 10 d at room temperature for maceration, filtered, concentrated and dried in a vacuum evaporator. Ethanolic extract of *T. arjuna* was dissolved in 1% DMSO [final concentration of the DMSO did not exceed 1% (v/v) and did not affect the cell proliferation] prepared in serum free RPMI medium and filtered by 0.3 mm syringe filter and stored.

HepG2 cell maintenance

Human hepatoma cell line (HepG2) was obtained from National Center for Cell Science, Department of Biotechnology, Pune, India. Cells were grown as monolayers in RPMI-1640 medium, supplemented with 10% (v/v) heat-inactivated FBS, antibiotics (penicillin 100U/mL, streptomycin 10 µg/mL) and 1mmol/L sodium pyruvate under standard conditions (37 °C) in a controlled humidified atmosphere containing 50 mL/L CO₂.

Cytotoxicity

Trypan blue exclusion method The cytotoxicity of the ethanolic extract of *T. arjuna* was assessed by cell viability using trypan blue exclusion method. For the determination of cell viability, cells were plated at the density of 5×10^4 cells/well and cultured for 48 h. The medium was replaced with serum-free medium [RPMI-1640 medium, supplemented with antibiotics (penicillin 100 U/mL, streptomycin 10 µg/mL, 1 mmol/L sodium pyruvate) and the cells were treated with various concentrations of *T. arjuna* (20, 40, 60, 80 and 100 mg/L) for 48 h and incubated with 1% DMSO as solvent control.

Lactate dehydrogenase leakage assay Lactate dehydrogenase (LDH) leakage assay was performed by the method of Grivell and Berry^[7]. One hundred microliters of conditioned media of control and *T. arjuna*-treated HepG2 cells was added to 1-mL cuvette containing 0.9mL of a reaction mixture to yield a final concentration of 1mmol/L pyruvate, 0.15 mmol/L NADH and 104 mmol/L phosphate buffer, pH7.4. After thoroughly mixed, the absorbance of the solution was measured at 340 nm for 45 s. LDH activity was expressed as µ moles of NADH used per minute per well.

Apoptosis

Light microscopic studies HepG2 cells were grown in 35 mm sterile petri plates and treated with *T. arjuna* extract at the concentrations of 60 and 100 µg/L for 48 h. The cells were then fixed for 5 min with 10% methanol/phosphate buffer saline (PBS) and morphological changes were observed under inverted microscope (Nikon, Japan).

Fluorescent microscopic studies Apoptotic morphology was studied by staining the cells with a combination of the fluorescent DNA-binding dye. After treatment with *T. arjuna* (60 and 100 mg/L for 48 h), cells were collected, washed and suspended in PBS. After stained with the equal mixture of acridine orange and ethidium bromide (each

dye was dissolved in 100 µg/mL of PBS), the cells were examined^[8]. The differential uptake of these two dyes allowed the identification of viable and non-viable cells under fluorescent microscope and the results were recorded.

DNA fragmentation DNA fragmentation was followed by the method of Chen *et al.*^[9]. The HepG2 cells were plated in a 60 mm culture dish at a density of 5×10^5 cells and treated with *T. arjuna* extract (60 and 100 mg/L) for 48 h. The cells attached at the bottom were scraped off and collected together with unattached cells by centrifuging at 1 500 r/min for 5 min at 4 °C. DNA was prepared from the pelleted cells. Briefly, the cells were lysed with lysis buffer and extracted with 2mL of phenol (neutralized with TE buffer, pH 7.5) followed by extraction with 1mL of chloroform and isoamyl alcohol in the ratio of 24:1. The aqueous supernatant was precipitated with 2.5 volume of ice-cold ethanol with 10% volume of sodium acetate at – 20 °C overnight. After centrifugation at 13 000 r/min for 10 min, the pellets were air-dried and resuspended with 50 µL of Tris–EDTA (TE) buffer. Equal quantities of DNA were electrophoresed in 1.8% agarose gel containing 0.5 mg/L of ethidium bromide. After electrophoresis, the gel was photographed under UV light.

Western blotting HepG2 cells (1×10^6 /mL) were treated with *T. arjuna* extract at the concentration of 60 and 100 µg/mL for 48 h at 37 °C. Cells were lysed with 10 µL of lysis buffer (150mmol/L Tris-HCl, 2 mmol/L N-ethylmaleimide, 2 mmol/L phenylmethyl sulfonyl fluoride, 4% sodium dodecylsulfate, 4% dithiothreitol, 20% glycerol, 0.01% bromophenol blue, 2 mol/L urea and 10mmol/L Na-EDTA) (pH 6.8). Cells were then sonicated three times for 10 s. Cell lysates were centrifuged at 15 000 r/min for 20 min at 4 °C. The protein levels were quantified using Biorad protein assay kit, USA. Equivalent amounts of proteins (60µg) were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane overnight at 25 mA. Membranes were blocked with blocking solution (2% BSA in PBS) for 1 h at 37 °C followed by incubation with primary antibodies (anti-p53 1:100; anticaspase-3 1:1 000) for 1 h under gentle agitation. The blots were washed three times and incubated with horse raddish peroxidase-conjugated antimouse IgG antibody for 1 h at 37 °C. Diaminobenzidine reagent was used to develop the immunoblots.

Estimation of glutathione

Total reduced glutathione (GSH) was determined by the method of Moron *et al.*^[10]. Five percent TCA was added to HepG2 (1×10^6 cells). The precipitate was removed by centrifugation. To an aliquot of the supernatant, 2mL of DTNB reagent was added to make a final volume of 3 mL. The absorbance was read at 412 nm against a blank containing TCA instead of sample. Aliquots of the standard solution were treated similarly. The amount of GSH was expressed as nmoles/10⁶ cells.

Statistical analysis

Values were expressed as mean ± SD. Difference between the drug-treated group and control group was analyzed with independent sample-‘t’ test using SPSS 7.5 student

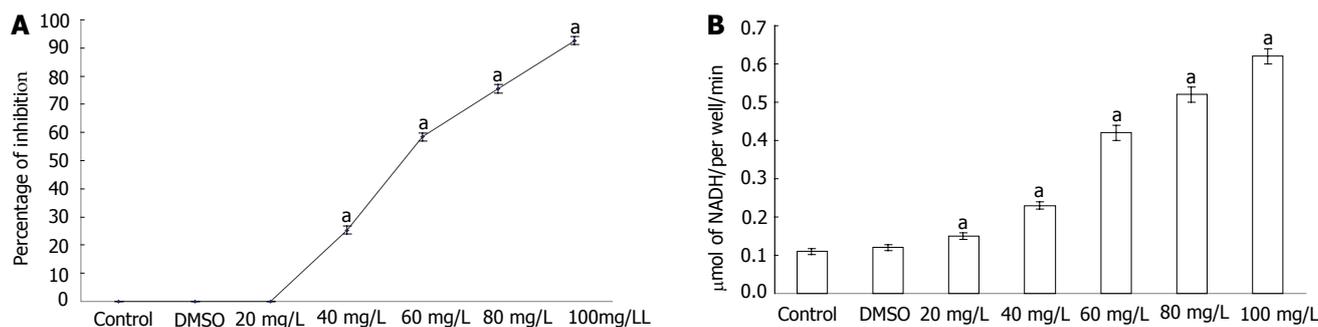


Figure 1 Viability (A) and LDH level (B) in control and HepG2 cells. Cells were exposed to different concentrations of *T. arjuna* extract for 48 h and percentage of viable and dead cells was determined using trypan blue exclusion method. Results were expressed as the percent of total cell number and the percentage of LDH leakage was analyzed. Values are mean \pm SD ($n = 6$). ^a $P < 0.05$ represents the statistical significance between control and *T. arjuna* treated HepG2 cells.

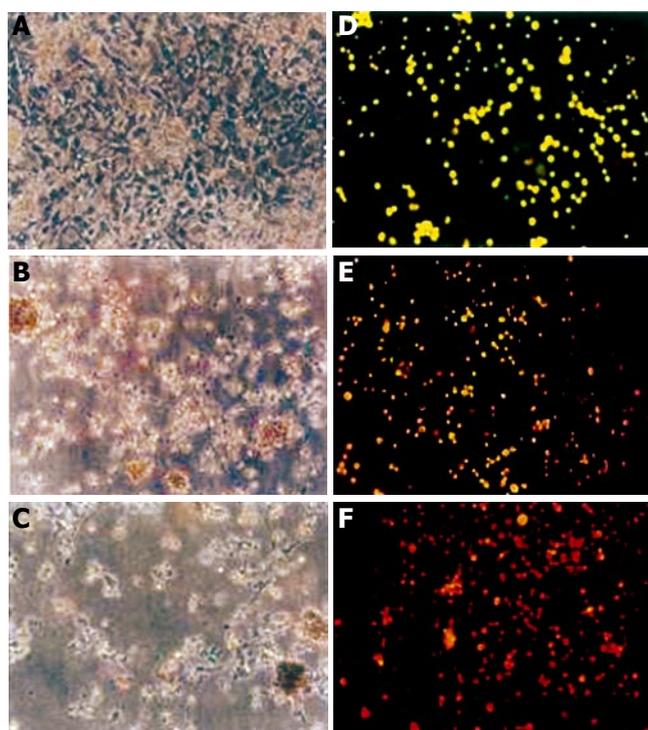


Figure 2 Morphological changes of HepG2 cells under light and fluorescence microscope. HepG2 cells were incubated for 48 h with *T. arjuna* extract. The medium was removed and the cells were rinsed and visualized under light microscope in control (A), 60 mg/L (B), 100 mg/L (C) of *T. arjuna* extract. Cells were stained with ethidium bromide and acridine orange and observed under fluorescence microscope in control (D), 60 mg/L (E) and 100 mg/L (F) of *T. arjuna* extract.

version. $P < 0.05$ was considered statistically significant.

RESULTS

Trypan blue exclusion method

The cytotoxicity of *T. arjuna* to HepG2 cells was examined by trypan blue exclusion and LDH leakage assay. Figure 1 (A) shows the viability of control and *T. arjuna*-treated (20, 40, 60, 80 and 100 mg/L) HepG2 cells. In the present study, the plant extract induced cytotoxicity to HepG2 cells in a concentration-dependent manner after 48 h of treatment. The results showed that treatment with *T. arjuna* could increase the number

of apoptotic cells and decrease the number of normal HepG2 cells. Apoptotic cells were detached from the surface and suspended in the medium.

Lactate dehydrogenase leakage assay

The levels of LDH released into the medium of control and *T. arjuna*-treated (20, 40, 60, 80 and 100 μ g/mL) HepG2 cells are presented in Figure 1 (B). LDH activities were significantly elevated after 48 h of exposure to *T. arjuna* extract in the medium when compared to the control.

Light microscopic observation

In *T. arjuna*-treated HepG2 cells, destruction of monolayer was observed, which was not seen in HepG2 cells not treated with it. *T. arjuna* treatment resulted in swelling and rounded morphology of HepG2 cells with condensed chromatin and their membrane also became crooked and vesicle shaped. Progressive structural alterations and reduction of HepG2 cell populations were observed [Figure 2 (A-C)].

Fluorescence microscopic observation

The morphological changes were observed in HepG2 cells treated with *T. arjuna* for 48 h. As shown in Figure 2 (D-F), normal live cells were bright green in color whereas *T. arjuna*-treated HepG2 cells were bright orange in color with condensed nuclei. Besides, normal nuclei showed chromatin with an organized structure, while apoptotic nuclei showed highly condensed chromatin in HepG2 cells treated with *T. arjuna*.

DNA fragmentation

In the control, no fragmentation was observed in agarose gel. However, the signal appeared in *T. arjuna* extract (60 and 100 mg/L) treated HepG2 cells showed the fragmented laddering pattern of DNA, indicating the characteristics of apoptotic cells (Figure 3).

Expression of p53 and caspase-3 proteins

T. arjuna extract treatment increased the band intensity of 53-ku protein in HepG2 cells compared to the control (Figures 4A and 4B). The accumulation of p53 protein induced apoptosis in *T. arjuna* extract-treated HepG2 cells. On the contrary, HepG2 cells treated with *T. arjuna* extract (60 and 100 mg/L) reduced the intensity of 32-ku protein

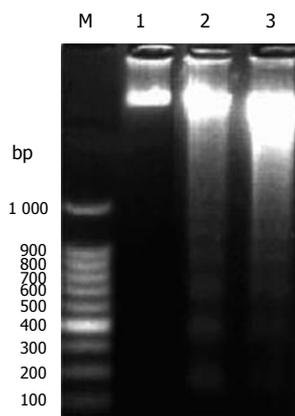


Figure 3 DNA fragmentation in HepG2 cells. HepG2 cells were treated with *T. arjuna* extract and incubated for 48 h at the concentration of 60 and 100 mg/L, respectively. DNA fragmentation was carried out as described in MATERIALS AND METHODS. Lane 1: 100 bp DNA marker; Lane 2: control HepG2 cells without any treatment; Lanes 3 and 4: HepG2 cells treated with *T. arjuna* extract at the concentration of 60 and 100 µg/mL, respectively.

and appearance of 17-kDa protein measured by densitometric analysis (Figures 4C and 4D). This results indicated that *T. arjuna* treatment stimulated the proteolytic cleavage of caspase-3 protein and initiated the apoptosis pathway in HepG2 cells.

GSH levels

The level of GSH content in control was 24.38 ± 1.37 n moles/ 10^6 cells. After treatment with *T. arjuna* (60 and 100 mg/L) the GSH level decreased to 18.57 ± 1.56 n moles/ 10^6 cells and 16.62 ± 1.46 n moles/ 10^6 cells, respectively. There was a significant difference between the *T. arjuna* extract-treated group and control group.

DISCUSSION

In the present investigation, *T. arjuna* extract markedly reduced the cell viability in a concentration-dependent manner. The suppression of cell proliferation induced by this extract may be due to the induction of cell death. Thus, the inhibitory activity of *T. arjuna* extract provides evidence for the *in vitro* cytotoxicity.

Recent studies suggest that LDH is a more reliable and accurate marker of cytotoxicity, because damaged cells are fragmented completely during the course of prolonged incubation with substances^[7]. In the present study, the LDH leakage increased significantly in *T. arjuna* extract-treated HepG2 cells when compared to the control cells. Extensive reports have documented on medicinal plant extract-induced cytotoxicity to cancer cells. It was reported that the acetone and methanolic extracts of *T. arjuna* inhibit the growth of transformed cells such as osteosarcoma cells (U2OS) and glioblastoma cells (U251) *in vitro* at the concentration of 30 and 60 mg/L^[11]. Thus, the inhibition of HepG2 cells strongly proves the cytotoxic nature of *T. arjuna* extract. Pasquini *et al.*^[12] also found that various fractions of *T. arjuna* inhibit the mutagenicity of 4-nitroquinoline-4-oxide in the salmonella/microsome test, suggesting that the inhibition may be due to the presence of antitu-

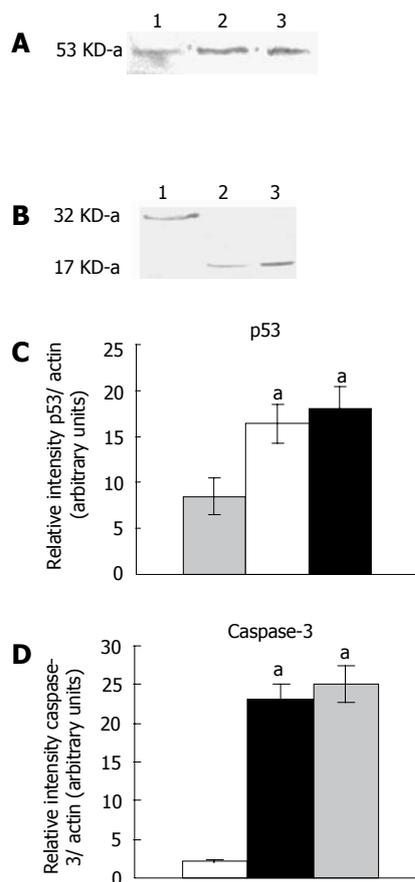


Figure 4 Expression of p53 and caspase-3 proteins. Western blotting analysis of p53 and caspase-3 in HepG2 cells cultured for 48 h in the presence and absence of *T. arjuna* extract at the concentration 60 and 100 mg/L was carried out. Cell lysates were prepared as described in MATERIALS AND METHODS, and resolved by 12% SDS-PAGE, immunoblotted and detected using specific antibodies which recognized p53 (A) and caspase-3 (B). Densitometric analysis of the p53 (C) and caspase-3 band (D) was performed. Each value is mean \pm SD of three observations. ^a $P < 0.05$ represents the statistical significance between control and *T. arjuna* extract-treated HepG2 cells.

mor promoters of the plant extract. Hence, the LDH leakage in HepG2 cells may be due to the cytotoxic nature of the plant extract and confirms its antitumor activity.

Light microscopic observation of *T. arjuna* extract-treated HepG2 cells at the concentrations of 60 and 100 mg/L after 48 h of exposure showed the typical morphological features of apoptosis in HepG2 cells. The morphological changes observed were reduction in cell volume, cell shrinkage, reduction in chromatin condensation and formation of cytoplasmic blebs. However, the control HepG2 cells had a higher confluency of monolayer.

Fluorescence microscopic study showed apoptosis in *T. arjuna* extract-treated HepG2 cells after exposed to the concentrations of 60 and 100 mg/L. In the present investigation, control HepG2 cells were stained green in color with acridine orange, because of cell membrane integrity. On the other hand, the drug-treated HepG2 cells were stained bright orange in color with ethidium bromide. This may be due to loss of cell membrane integrity and apoptotic nature of the plant extract.

Apoptotic cells often produce a unique ladder composed of nucleotide fragments at an interval of 180-200

base pairs, which can be visualized by DNA-agarose gel electrophoresis. It is generally assumed that the toxicity of antitumor drugs is the consequences of their ability to cause genomic DNA damage in cancer cells^[13]. In this context, Bai and Cederbaum^[14] reported that many chemotherapeutic drugs, including Vp16 and mitomycin C, induce cells to undergo apoptosis through damage of nuclear DNA. In the present study, DNA ladders appeared in *T. arjuna* extract-treated HepG2 cells after exposed to the concentrations of 60 and 100 µg/mL for 48 h. However, the control HepG2 cells did not show any DNA fragmentation. In general, cytotoxic drugs induce a massive breakage of DNA into oligonucleosome fragments. The degradation of DNA down to oligonucleosomal fragments is a late event of apoptosis^[15]. Thus, the *T. arjuna* extract induces DNA damage in HepG2 cells and thereby causes apoptosis.

It is well known that p53 acts as a guardian of the genome, and is one of the major factors controlling cell proliferation, growth and transformation. The p53 tumor suppressor gene is mutated in over 50% of human cancers, and the oncogenic activity of p53 mutation is due to its ability to interfere with p53-dependent apoptosis by a dominant negative mechanism^[16]. Tumor suppressor gene p53 is one of the critical genes regulating the onset of DNA replication around G1/S boundary. Also, p53-mediated tumor suppression appears to be critical for therapeutic potential in the treatment of tumors^[17]. p53 is implicated in cell cycle control, DNA repair and apoptosis^[18,19]. Thus, apoptotic modulators may be promising agents for treatment of HCC. p53 contributes to apoptosis induced by a variety of cellular stresses, including DNA damage, oxidative stress and chemotherapeutic drugs^[20].

Activation of p53 is often deduced with natural chemotherapeutic agents and p53 negative tumors are generally less sensitive or even insensitive to these agents. In the present study, *T. arjuna* extract-treated HepG2 cells showed upstream regulation of p53 protein expression after exposed to the concentrations of 60 and 100 mg/L for 48 h. Hence, *T. arjuna* extract may possibly enhance the susceptibility of HepG2 cells to apoptosis by attenuating the tumor suppressor protein.

In cells undergoing apoptosis, there is a activation of proteases known as caspases, which have an obligatory cystein residue within the active site and cleave peptides adjacent to an aspartic acid residue^[21]. Caspase cascade has been demonstrated to be involved in apoptotic signal transduction and execution^[22]. Human caspases 1-10 have been described and activation of the caspase cascade is involved in chemical-induced apoptosis^[23], including degradation of DNA repair enzyme poly ADP ribosepolymerase^[24] and DNA-dependent protein kinase as well as cleavage of chromatin at inter-nucleosomal sites mediated by caspase-activated DNase^[25]. Generally, caspases are present as inactive proenzymes, most of which are activated by proteolytic cleavage. Caspases-8, -9 and -3 are situated at pivotal junctions in apoptotic pathways^[26]. Caspase-3 may then cleave vital cellular proteins or activate additional caspases by proteolytic cleavage. In the present investigation, *T. arjuna* extract-treated HepG2 cells showed a low intensity of 32-ku protein band and 17-ku protein band

was observed. According to Busihardjo *et al*^[27], caspase-3 usually exists as an inactive procaspase-3 that becomes proteolytically activated by multiple cleavages of its 32-ku precursor to generate the 20/11 or 17/11 ku active forms in cells undergoing apoptosis. Thus, the appearance of 17-ku protein may be one of the active forms of caspase-3 protein. It may be due to proteolytic cleavage of inactive caspase-3 induced by the plant extract. This occurs before the further activation of caspase-3-mediated apoptosis. Recent studies have also suggested that the proteolytic degradation of specific substrates is responsible for many of the morphological features of apoptosis^[28,29].

It was reported that certain products from plants can induce apoptosis in neoplastic cells but not in normal cells^[30,31]. Studies have shown that cytotoxic effect of the phenolic compounds on different tumors is mediated through apoptosis. For instance, gallic acid selectively induces cell death in various transformed cell lines such as PLC/PRF/5 (human hepatoma), HL-60, RG (human promyelocytic leukemia) and P-388D₁ (mouse lymphoid neoplasma) the^[32].

It has been reported that several putative compounds isolated from *T. arjuna* such as tannic acid, especially flavonoid (luteolin) are potent antitumor promoters and inhibitors of a series of solid tumors and leukemia^[33,34]. Nagpal *et al*^[11] have reported that the growth of osteosarcoma (U2OS) and glioblastoma (U251) is inhibited after treated with *T. arjuna* extract, suggesting that the induction of apoptosis in HepG2 cells may be due to the antitumor property of *T. arjuna* extract.

Numerous data indicate that intracellular oxidative metabolites play a significant role in the regulation of apoptosis. The reduced tripeptide GSH is a hydroxyl radical and a singlet oxygen scavenger, participating in a wide range of cellular functions such as protein and DNA synthesis, intermediary metabolism and transport^[35]. The maintenance of GSH levels and the reducing environment of cells are crucial^[36]. Depletion of GSH leads to increased accumulation of lipid peroxides and loss of cell viability^[37]. It is known that the toxicity of antitumor drugs may largely depend on the intracellular level of reduced GSH. Because GSH is the main antioxidant system in cells, a possible explanation is that GSH depletion facilitates reactive oxygen species (ROS) accumulation in cells treated with antitumor drugs^[38], which in turn increases their lethality. In the present study, the level of GSH significantly decreased in *T. arjuna* extract-treated HepG2 cells at the concentration of 60 and 100 µg/mL, indicating that the decrease in GSH levels may initiate redox imbalance in HepG2 cells and subsequently induces apoptosis. Depletion of GSH has been described for several agents such as oxidative and alkylating agents in various cell types^[39]. Cells with reduced GSH levels either undergo apoptosis or become more sensitive to various death inducing agents^[40,41] Liu *et al*^[42] also found that *Salvia miltiorrhiza* inhibits human hepatoma HepG2 cell growth and induces apoptosis involving intracellular GSH depletion. Hall^[43] has demonstrated that onset of apoptosis is associated with a fall of intracellular GSH in different cellular systems. Loss of GSH is tightly coupled with a number of downstream events in apoptosis^[44].

Studies in a variety of cell types suggest that cancer

chemotherapeutic drugs induce tumor cell apoptosis in part by increasing the formation of ROS. According to Simizu *et al.*^[45], some anticancer agents, including vinblastin and camptothecin, induce cell apoptosis with the generation of ROS. Previous reports suggest that quercetin and related phenolic compounds containing a catechol moiety in their chemical structures are oxidized under certain *in vitro* conditions, resulting in lipid peroxidation^[46, 47]. Accordingly, it is speculated that *T. arjuna* extract may induce ROS production in HepG2 cells and subsequently causes apoptosis. However, ROS may not necessarily be the direct factor to cause apoptosis induced by the drug, but intracellular ROS may modulate the genes involved in apoptosis, which may regulate apoptosis. Thus, *T. arjuna* extract-induced oxidative stress is upstream of signaling events that might alter the pro and antiapoptotic balance in HepG2 cells. Phenolic compounds are generally known to show not only their antioxidant effects but also pro-oxidant actions under *in vitro* conditions. Hence, it is possible that treatment of HepG2 cells with *T. arjuna* extract can deplete the GSH levels and promote oxidation induction, which switches the mode of death via apoptosis. Therefore, the cytotoxic action of this drug may be attributed to its pro-oxidant action on the cells. This may be able to account for the discrepancy between *in vitro* cytotoxicity and *in vivo* antitumor activities of *T. arjuna* extract.

In conclusion, *T. arjuna* extract has profound effects on human hepatoma cellline HepG2 and exhibits its cytotoxicity to these cells and the cell death is mediated by apoptosis. The mechanism of apoptosis may be accumulation of p53 protein and proteolytic cleavage of caspase-3 protein. In addition to these, GSH depletion may also play a role in apoptosis through redox imbalance in HepG2 cells. Thus, our results provide the basis for further *in vivo* and clinical research.

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Identification of differently expressed genes in human colorectal adenocarcinoma

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Abstract

AIM: To investigate the differently expressed genes in human colorectal adenocarcinoma.

METHODS: The integrated approach for gene expression profiling that couples suppression subtractive hybridization, high-throughput cDNA array, sequencing, bioinformatics analysis, and reverse transcriptase real-time quantitative polymerase chain reaction (PCR) was carried out. A set of cDNA clones including 1260 SSH inserts amplified by PCR was arrayed using robotic printing. The cDNA arrays were hybridized with fluorescent-labeled probes prepared from RNA of human colorectal adenocarcinoma (HCRAC) and normal colorectal tissues.

RESULTS: A total of 86 genes were identified, 16 unknown genes and 70 known genes. The transcription factor Sox9 influencing cell differentiation was downregulated. At the same time, Heat shock protein 10 KDis downregulated and Calmoulin is up-regulated.

CONCLUSION: Downregulation of heat shock protein 10 KD lost its inhibition of Ras, and then attenuated the Ras GTPase signaling pathway, increased cell proliferation and inhibited cell apoptosis. Down-regulated transcription factor Sox9 influences cell differentiation and cell-specific gene expression. Down-regulated Sox9 also decreases its binding to calmodulin, accumulates calmodulin as receptor-activated kinase and phosphorylase kinase due to the activation of PhK.

INTRODUCTION

The lifetime risk of colorectal cancer in the general population is about 5-6% and about 30-40% of patients have cancer-related deaths^[1]. It is of great importance to elucidate the mechanisms involved in human colorectal gene carcinogenesis at cellular and molecular levels. It has been demonstrated that colorectal tumor undergoes multiple and sequential morphological and molecular changes^[2]. In the process of the disease, cell division, differentiation and apoptosis are related to the expression disorder of a group of genes and protein interaction. Oncogenes such as myc, K-ras, src, and erbB2 are activated, while the suppressive cancer genes p53, DCC, APC lose their activation and mismatch repair leads to microsatellite instability^[3-9]. However, the exact gene expression profiling for human colorectal adenocarcinoma (HCRAC) is still unclear.

Suppression subtractive hybridization (SSH) techniques based on suppression polymerase chain reaction (PCR) can identify the gene expression profiling^[10]. We have described recently the successful use of SSH using HCRAC and normal colorectal tissues^[11]. Combined with cDNA microarray techniques, the gene expressing profiling of HCRAC can be obtained with four additional pairs of normal colorectal and HCRAC tissues. In this study, 86 genes were identified. According to the results of sequencing and bioinformatics analyses, three genes were selected, which differed strongly between HCRAC and normal colorectal tissues, and four genes were evaluated in 10 pairs of matched normal colorectal and HCRAC tissues by QRT-PCR technique.

Table 1 QRT-PCR parameters

| Gene | Primer | Sequence | Length of primer (bp) | Length of product (bp) | Location of product | |
|----------------|---------------|--|-----------------------|------------------------|---------------------|-------|
| HSE1 | Forward prime | 5'-TGGCAGGACAAGCGTTTAGA-3' | 20 | 66 | 43-108 | |
| | Reverse prime | 5'-CAGCACTCCTTTCAACCAATACTC-3' | 24 | | | |
| | TaqMan probe | 5'->FAM AGTTTCTTCCACTCTTTG Quencher < 3' | 18 | | | 64-81 |
| SOX9 | Forward prime | 5'-AGCGACGTCATCTCCAACATC-3' | 21 | 49 | 1235-1 | |
| | Reverse prime | 5'-GTTGGGCGGCAGGTACTG-3' | 18 | | | 252 |
| | TaqMan | 5'->FAM CCTTCGATGTCAACGAGT Quencher < 3' | 18 | | | |
| CaM | Forward prime | 5'-GTTGAGCGAGGCAAATGGAT-3' | 20 | 62 | 3098-3 | |
| | Reverse prime | 5'-TCCTGGCAACAGTGCATCA-3' | 20 | | | 161 |
| | TaqMan | 5'->FAM TCGATATTCAGATGGGC Quencher < 3' | 18 | | | |
| β -Actin | Forward prime | 5'-CTGGCACCCAGCACAATG-3' | 18 | 93 | 1058-1 | |
| | Reverse prime | 5'-GGACAGCGAGGCCAGGAT-3' | 18 | | | 075 |
| | TaqMan | 5'->FAM ATCATTGCTCCTCTGAG Quencher < 3' | 18 | | | |

MATERIALS AND METHODS

Tissue sample

Normal colorectal and HCRAC tissue samples from HCRAC patients in West China Hospital were snap-frozen in liquid nitrogen immediately after surgery and stored at -80 °C. Ten pairs of HCRAC and normal colorectal tissues were used. All the tumor samples containing >50% of tumor cells were confirmed as colorectal adenocarcinoma according to the result of pathologic diagnosis.

RNA extraction

Isolation of RNA was performed using the TRIzol method (Invitrogen) according to manufacturer's instructions. RNA from each sample was assessed by visualization of the 28S/18S ribosomal RNA ratio on 1% agarose gel. RNA yield was determined by measuring the absorbency at 260 nm.

Suppression subtractive hybridization

PCR-based cDNA subtraction method and PCR-select subtraction method (Clontech Laboratories) were performed using the SMART PCR cDNA synthesis as described previously^[10,11]. Total RNA was isolated from HCRAC and normal rectum tissues, respectively, and reversely transcribed into single-strand cDNAs with reverse transcript enzyme superscript II. Double-strand cDNA was then synthesized and digested with restriction enzyme *RsaI*, resulting in fragments with a different size of 400-600 bp. HCRAC cDNAs were divided into two groups and ligated to the specific adaptors 1 and 2R, respectively. After HCRAC cDNAs were hybridized with normal rectum cDNA twice for 10 h at 68 °C and the resulting cDNAs were amplified with nested PCR twice, the amplified cDNAs containing enriched differently expressed transcripts were cloned into plasmid vector arms of T/A. The ligated cDNAs were transformed into *E. coli* strain JM109.

cDNA microarrays

A cDNA gene chip containing 1260 SSH clones was

made. The inserted fragments in the pMD-18T (TaKaRa Company) were amplified with PCR using primers from the franking cloning site. Then the amplified PCR products were spotted onto silylated slides (CEL Associates, Houston, TX, USA) using a Cartesian PixSys 7500 motion control robot (Cartesian Technologies, Irvine, CA, USA) fitted with ChipMaker Micro-Spotting Technology (TeleChem International, Sunnyvale, CA, USA). The total RNA from the four pairs of samples taken from grade A patients was used to prepare cDNA fluorescent probes for hybridization to microarray. Probes were prepared by reverse transcription of total RNA in the presence of either Cy-5 or Cy-3 labeled dUTP (Amersham Pharmacia) using superscript II (Gibco-BRL). The two color probes were then mixed, and the denatured probe mixtures were applied onto the pre-hybridized chip under a cover glass. The chips were hybridized at 42 °C for 15-17 h and scanned with a ScanArray 3000 (GSI Lumonics, Billerica, MA, USA) at two wavelengths to detect emission from both Cy3 and Cy5. The acquired images were analyzed using ImaGene 3.0 software (BioDiscovery, Inc., Los Angeles, CA, USA). The intensities of each spot at the two wavelengths represented the quantity of Cy3-dUTP and Cy5-dUTP, respectively, hybridized to each spot. Genes were identified as differently expressed, if the absolute value of the natural logarithm of the ratios was >0.69. To minimize artifacts arising from low expression values, only genes with raw intensity values for both Cy3 and Cy5 >800 counts were chosen for differential analysis. The clustering algorithm separated tumor and normal tissues into different clusters. We used an algorithm based on the hierarchical clustering algorithm to organize the data matrix of the four colorectal adenocarcinoma samples in a binary tree.

Sequence and data analysis

Sequencing was carried out for the cDNA clones using the Thermo Sequenase fluorescent labeled primer cycle sequencing (Amersham Pharmacia Biotech, UK). CR-r6d4cts were run on SequaGel (Biozyme, Oldendorf, Germany) and the sequences were analyzed by a LiCOR

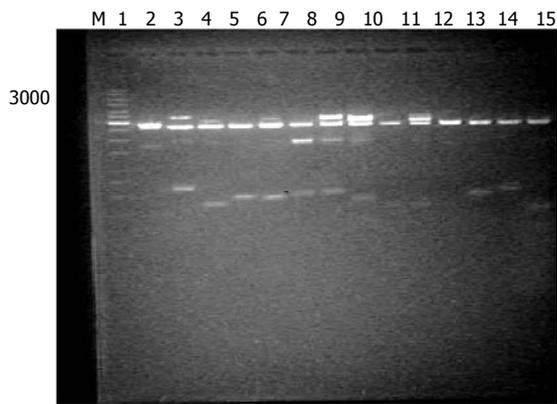


Figure 1 Digestion plasmids of *EcoRI/HindIII*. Lane M: DNA size markers; 1-15 lanes: Fragment of about 500 bp after *EcoRI/HindIII* digestion.

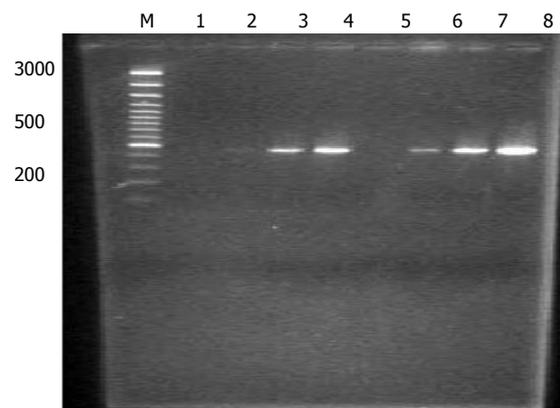


Figure 2 Reduction of G3PDH after PCR-select subtraction. M: DNA size markers; 1-4 lanes: Subtracted PCR products as template, G3PDH3' and 5' as primers at cycles 18, 23, 28, 33; 5-8 lanes: PCR products not subtracted as template, G3PDH3' and 5' as primers at cycles 18, 23, 28, 33.

sequencer (MWG Biotech, Ebersberg, Germany). The qualified expressed sequence tags (ESTs) were referred to the ESTs containing less than 3% ambiguous bases and longer than 100 bp. These ESTs were subjected to BLAST analysis. The EST was considered as part of a known gene, if the homology to the known gene was over 80%. The EST was considered as part of a known EST, if it shared 95% homology with at least 100 bp of human EST. The EST with no match to human EST was considered as a novel EST.

cDNA synthesis and real-time PCR

The total RNA from 10 pairs of patients was treated with RNA-free DNAase I (TaKaRa Company) to remove any genomic DNA contamination, then subjected to reverse transcription using M-MuLV reverse transcriptase (Qiagen Company, Germany).

PCR primers were designed using Primer Express 1.5 (PE Company) with the following parameters: length of the amplicon was between 50 and 150 bp, T_m of the primers was between 58 °C and 60 °C, and span of an intron (Table 1). Real time quantitative (RTQ) PCR was performed using Taqman MGB reagent kit (Qiagen Company, Germany) according to the manufacturer's instructions. PCR was performed on an ABI Prism 700 fluorescent quantitative PCR.

RESULTS

Suppression subtractive hybridization

Generation of subtracted cDNA populations SSH was performed in human rectum adenocarcinoma and normal rectum tissues from one patient. A total of 1 260 clones were obtained by SSH. To evaluate the quality of the libraries, plasmid DNA isolated from 36 clones was digested with restriction enzyme, and agarose gel electrophoresis showed that all plasmids contained 400-600 bp fragments (Figure 1). Sequence analysis of 31 clones demonstrated that 28 fragments were differentially homologous to known genes in the GenBank (acquired gene accession number and dbEST number: Accn

BM360856-BM360883).

Analysis of subtraction efficiency To estimate the efficiency of subtraction in subtractive cDNA library, the amount of cDNA for G3PDH and oncogene *c-myc* was analyzed using subtracted and unsubtracted PCR products as templates. Eighteen, 23, 28 and 33 cycles of PCR reactions were performed in a total reaction volume of 30 μ L. The result demonstrated that the non-specifically expressed housekeeping gene G3PDH was greatly decreased in the reaction (Figure 2). In contrast, the specifically expressed oncogene *c-myc* was greatly increased in the reaction (Figure 3). These results indicated that the present suppression subtractive cDNA library was highly efficient in enriching the highly expressed genes in HCRAC.

cDNA microarray

The cDNA inserts of each clone were amplified with PCR and spotted on a microarray using robotic printing. Multiple housekeeping genes and randomly selected cDNAs were also printed on the same array to serve as internal controls. The cDNAs from cancer tissue of four patients were labeled with cyanine5 (Cy5), and the cDNAs from normal colon tissue of four patients were labeled with cyanine3 (Cy3). The microarrays were hybridized with cDNA probes labeled with fluorochrome in the forward direction as group 1 (Figure 4A). Then, the cDNAs from cancer tissue of four patients were labeled with Cy3, and the cDNAs from normal colon tissue of four patients were labeled with Cy5. The microarrays were hybridized with cDNA probes labeled with fluorochrome by reverse direction as group 2 (Figure 4B). Red and green fluorescence indicated greater relative expression in the tissues of HCRAC and normal colon, yellow fluorescence indicated equal expression. According to the changes in signal intensity, a total of 143 ESTs were identified. Fifty-four ESTs were differently expressed in group 1, 37 ESTs were upregulated and 17 ESTs were downregulated by forward hybridization. ESTs were differently expressed in group 2 by reverse hybridization, 49 ESTs were upregulated, 40 ESTs were downregulated. The identified

Table 2 Differently expressed genes in subtracted cDNA populations

| | Name of gene | Accession number | cDNA | cDNA array ratio | |
|--|---|--|------------------------------|--|----------------------------|
| | | | | Group 1 | Group 2 |
| Proto-oncogene and tumor suppressor genes | ITGA6: integrin, alpha 6 | ck570352 | C60 | | 2.277 |
| Ion channels proteins | ATP1B1: ATPase, Na ⁺ /K ⁺ transporting, beta 1 polypeptide | ck570329 | C8 | | 0.352 |
| | CALM1: calmodulin 1 (phosphorylase kinase, delta) | ck570304, ck570314 | a50, b19 | 3.265, 1.856 | 0.344 |
| Cell cycle proteins | PTMA: prothymosin, alpha | ck570346 | C47 | | 2.090 |
| | BTG1: B-cell translocation gene 1, anti-proliferative DKFZp451J0118: taxilin | ck570300 | a21 | 2.058 | |
| | CAPZA1: capping protein (actin filament) muscle Z-line, alpha 1 | ck570339, ck570343 | c32, c41 | | 0.472, 0.493 |
| Cell frame and movement | TBDN100: transcriptional coactivator tubedown-100 | ck570351 | c58 | | 2.211 |
| | K-ALPHA-1: tubulin, alpha, ubiquitous | ck570353 | C61 | | 2.281 |
| | TUBB2: tubulin, beta, 2 | ck570366 | C91 | | 3.21 |
| | CAPZA1: capping protein (actin filament) muscle Z-line, alpha 1 | ck570343 | C41 | | 0.493 |
| Apoptosis related proteins | Signal K-ALPHA-1: tubulin, alpha, ubiquitous | ck570339 | C32 | | 0.472 |
| | SRPRB: signal recognition particle receptor, B subunit | ck570312 | b12 | 1.540 | 0.425 |
| transmitting related proteins | PERQ1: PERQ amino acid rich, with GYF domain 1 | ck570299 | a15 | 0.486 | |
| Synthesis and translation related proteins | ZNF403: zinc finger protein 403 | ck570360 | C85 | 3.197 | 3.197 |
| | SOX9: SRY (sex determining region Y)-box 9 (campomelic dysplasia, autosomal sex-reversal) | ck570305, ck570306, ck570361 | b2, b3, c86 | 0.426, 0.489 | 2.344, 1.593, 3.391 |
| | SET: SET translocation (myeloid leukemia-associated) | ck570344 | c43 | | 2.447 |
| | Bit1: Bcl-2 inhibitor of transcription | | | | |
| | EIF3S6: eukaryotic translation initiation factor 3, subunit 6 48 KD | ck570355, ck570311 | c73, b11 | 0.660 | 2.666, 2.091 |
| | EIF4E: eukaryotic translation initiation factor 4E | | | | |
| | NCL: nucleolin | ck570336 | c16 | | 0.392 |
| Metabolism related proteins | RPL7: ribosomal protein L7 | ck570367 | c92 | | 4.67 |
| | RPS24: ribosomal protein S24 | ck570290 | a3 | 0.359 | |
| | RPS25: ribosomal protein S25 | ck570297 | a13 | 0.457 | |
| | COX7C: cytochrome c oxidase subunit VIIc | ck570349 | c55 | | 2.202 |
| | FABP1: fatty acid binding protein 1, liver | ck570315 | b20 | 1.860 | 0.580 |
| | SAT: spermidine/spermine N1-acetyltransferase | ck570324 | b39 | 2.807 | 0.547 |
| | SCD: stearyl-CoA desaturase (delta-9-desaturase) | ck570335 | c13 | | 0.375 |
| | PAFAH1B2: platelet-activating factor acetylhydrolase, isoform Ib, beta subunit 30 KD protein 1 | ck570357 | c81 | | 3.098 |
| Immunity and stimulation related proteins | HSPE1: heat shock 10 protein 1 (chaperonin 10) | ck570292 | a7 | 0.421 | |
| | | ck570307, ck570308, ck570310, ck570347 | b6, b7, b9, c52 | 0.562, 0.567, 0.649 | 3.191, 2.686, 2.532 |
| | HSPD1: heat shock 60 KD protein 1 (chaperonin) | ck570356 | c77 | | 2.132, 2.786 |
| | IGHG1: immunoglobulin heavy constant gamma 1 (G1m marker) | ck570363, ck570321 | c90, b5-1 | 0.493 | 4.667, 2.830 |
| | IFITM3: interferon induced transmembrane protein 3 (1-8 U) | ck570309 | b8 | 0.634 | 3.021 |
| Cell receptor | PSMA7: proteasome (prosome, macropain) subunit, alpha type, 7 | | | | |
| | PIGR: polymeric immunoglobulin receptor | ck570301, ck570303, ck570313, ck570316, ck570318, ck570319 | a28, a39, b13, b21, b24, b40 | 2.309, 2.552, 1.554, 1.869, 1.982, 2.942 | 0.330, 0.505, 0.394, 0.662 |
| Others | C6orf62: chromosome 6 open reading frame 62 | ck570289 | a2 | 0.236 | |
| | IRA1: likely ortholog of mouse IRA1 protein | ck570291 | a4 | 0.402 | |
| | MRNA: cDNA DKFZp686P07216 (from clone DKFZp686P07216) | ck570296 | a12 | 0.450 | |
| | DACH1: dachshund homolog 1 (<i>Drosophila</i>) | ck570298 | a14 | 0.482 | |
| | TXNIP: thioredoxin interacting protein | ck570317 | b23 | 1.959 | 0.297 |
| | LOC376745: AG1 | ck570328 | c5 | | 0.336 |
| | Transcribed sequence with strong similarity to protein prf:0512543A (<i>H. sapiens</i>) 0512543A oxidase II, cytochrome | ck570333 | c12 | | 0.373 |
| | C14orf112: chromosome 14 open reading frame 112 | ck570337 | c19 | | 0.414 |
| | Transcribed sequence with moderate similarity to protein sp:P18124 (<i>H. sapiens</i>) RL7_HUMAN 60S ribosomal protein L7 | ck570348, ck570354 | c53, c71 | | 2.152, 2.648 |
| | F L J32421: hypothetical protein FLJ32421 | ck570358 | c82 | | 3.127 |
| | C20orf45: chromosome 20 open reading frame 45 | ck570364, ck570322, ck570327 | c50, b38, b44 | 2.665, 2.942 | 2.104, 0.368, 0.590 |
| | CDNA clone MGC:62026 IMAGE:6450688, complete cds | ck570330 | c10 | | 0.368 |

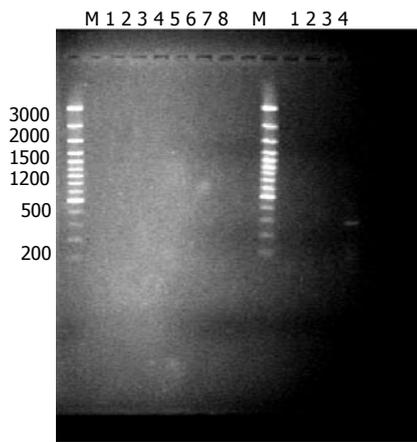


Figure 3 Increased *c-myc* after PCR-select subtraction. M: DNA size markers; 1-4 lanes (left): PCR products not subtracted as template, *c-myc* 3' and 5' as primers at cycles 18, 23, 28, 33; 5-8 lanes: No PCR products subtracted as template, *c-myc* 3' and 5' as primers at cycles 18, 23, 28, 33; 1-4 lanes (right): PCR products subtracted as template, *c-myc* 3' and 5' as primers at cycles 18, 23, 28, 33.

43 ESTs representing 3.41% of total genes examined were differently expressed in the two groups (Table 2).

DISCUSSION

Controlling reliability of SSH

HCRAC is a multi-gene disease similar to other malignant tumors^[1]. Although the expression of some genes such as APC, DCC and p53 is related with the occurrence of HCRAC, the underlying mechanisms and the whole gene expression profile involved in the pathogenesis of HCRAC are still unclear^[5-9]. In this study, according to the SSH technique based on suppression PCR^[10], the cDNA subtractive library of HCRAC was constructed. The subtraction efficiency was estimated by PCR analysis. The amount of G3PDH was relatively decreased and the amount of *c-myc* was relatively increased. Thirty-six randomly selected clones were sequenced. Bioinformatics analyses showed human ESTs and dbEST with their accession numbers in the GenBank^[11].

cDNA microarray and quantitative PCR

SSH combined with cDNA microarray or real-time-PCR technique has been used as an efficient method to identify differently expressed genes in breast cancer cells, renal cells, colon cancer cells, non-metastatic and metastatic cancer cells, lung cancer cells, bronchial epithelial cells, fibroblast growth factor 2-transformed endothelial cells^[12-19]. It was reported that some differently expressed tissue-specific genes have been identified in nasopharyngeal epithelial tissue and two novel full-length genes have been isolated in human glioma specimens^[20,21].

Cancer is a highly variable disease with multiple heterogeneous genetic and epigenetic changes. Functional studies are essential to understand the complex polymorphisms of cancer. Microarray is a new powerful tool for studying the molecular basis of interaction. This technique makes it possible to examine the expression of thousands of genes simultaneously^[23]. To further confirm

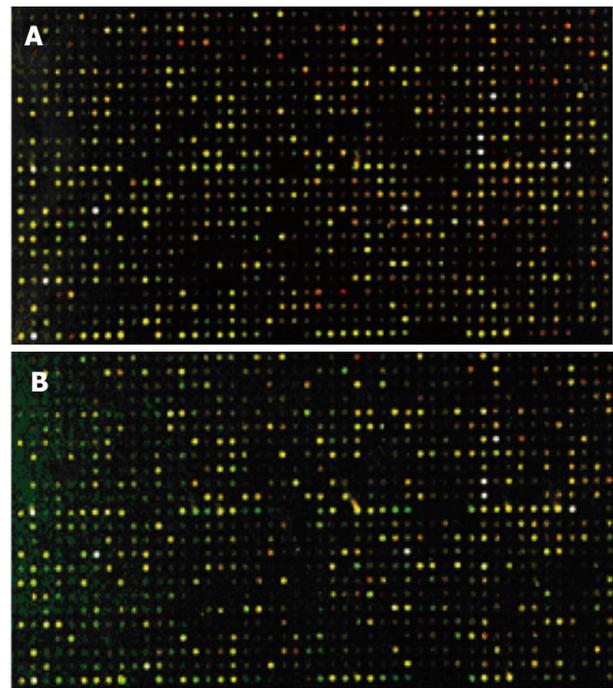


Figure 4 cDNA microarray hybridized with fluorescent labeled probes prepared from HCRAC (A) and total RNA from normal colorectal tissue (B).

the different expression of genes identified in subtractions in the present study, four pairs of samples of patients were chosen for cDNA microarray analysis. The results of the forward and reverse hybridizations were identical.

Both thermodynamic and physical parameters are known to influence hybridization intensities on DNA microarray, and they may fail in discriminating highly similar genes within a gene family during heterologous probe hybridization. Among the techniques available to validate the DNA microarray data, we chose quantitative real-time RT-PCR (qRT-PCR), since it could offer confirmatory quantitative results under stringent conditions. qRT-PCR can be used for measuring low concentrations of mRNA, and is highly accurate, reproducible, and amenable to high throughput analysis^[23-31]. Three differently expressed ESTs were selected and their expression was analyzed by reverse transcriptase real-time quantitative PCR in the ten pairs of patients. The expression of the four cDNA fragments tested displayed the expected different patterns of expression (Figure 5).

Global molecular change in carcinogenesis

Carcinogenesis is a complex process in which many molecules are changed at transcriptional and translational levels, and then manifested as structural and functional changes of cancer cells. In forward microarray hybridization, eukaryotic translation initiation factor 3 and SRY Sox are downregulated. Furthermore, heat shock proteins 10 and 60 KD, ribosomal protein S 17, ribosomal protein S 25, ribosomal protein S 28, ribosomal protein L7, tubulin alpha and beta, E-cadherin and integrin alpha 6 proteasome are transiently downregulated. The up-regulated calmodulin 1, nuclear gene encoding mitochondrial protein, protease, ATPase, Na⁺/K⁺ beta

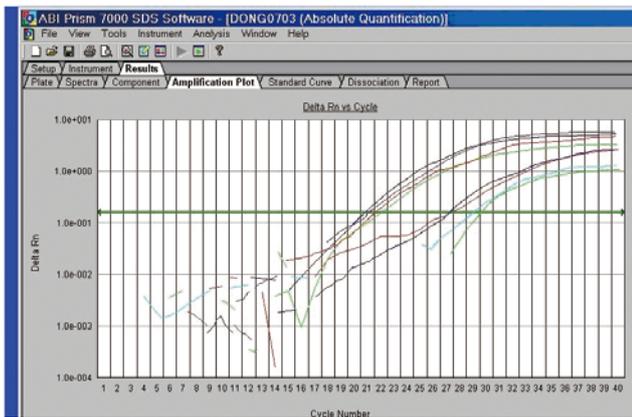


Figure 5 Results of QRT-PCR. Blue/brown/: β -actin N/ β -actinT; green/bright green: CaMT/CaMN; green/red: Hsp10T/Hsp10N; blue: Sox-9N/Sox-9T.

transporting polypeptide, Zinc finger protein, and putative ribosomal RNA apurinic site-specific lyase have been demonstrated in forward microarray hybridization. The results of reverse microarray hybridization coincided with it.

Translation initiation in eukaryotes is a rate-limiting step in protein synthesis. It is a complex process involving many eukaryotic initiation factors (eIFs). Altering the expression level or the function of eIFs may influence the synthesis of some proteins and consequently cause abnormal cell growth and malignant transformation^[32]. Ribosomes that catalyze synthesis of proteins consist of a small 40S subunit and a large 60S subunit. These subunits are composed of four RNA species and approximately 80 structurally distinct proteins^[33]. The down-regulated tubulin has been observed. This experiment renders further support to the hypothesis that activation of both the transcriptional and posttranscriptional machinery may constitute an integral part of the mechanism underlying carcinogenesis.

Down-regulated E-cadherin and integrin alpha 6 can decrease cancer cell adhesion and increase its invasion. Up-regulated nuclear genes encoding mitochondrial protein, ATPase, Na⁺/K⁺ beta transporting polypeptide reflect that cancer cells need enough energy to grow.

Signal transmission of carcinogenesis

We observed that Sox9 was strongly down-regulated (0.426-fold). Sox, a high-mobility-group domain containing transcription factor, with a DNA-binding domain similar to that of the mammalian testis-determining factor SRY, is a key transcription factor that is essential for chondrocyte differentiation and chondrocyte-specific gene expressions^[34,35]. Sox9 has been demonstrated to be a "master regulator" gene that controls distinct pathways of mesenchymal differentiation. Sox9 is a target of cAMP signaling and phosphorylation of Sox9 by protein kinase A (PKA) enhances its transcriptional and DNA-binding activity^[36-38]. Sox9 harbors a number of highly conserved regions, including two domains required for maximal trans-activation. The heat shock protein HSP70 recognizes a specific region of Sox9 with unknown function which may facilitate the assembly of multi-protein complexes

at promoter enhancer regions. The Sox9 HMG domains carry two nuclear localization signals (NLSs). The N-terminal NLS binds to calmodulin while the C-terminal NLS binds to importin beta^[39].

We also found that heat shock protein 10 KD (chaperonin 10) was downregulated (0.562-fold). Heat shock protein 10 KD can reduce myocyte death by its mitochondrial function or by interacting with cytoplasmic signaling pathways. The Ras GTPase signaling pathway indicates that inhibition of Ras is required for the protection by HSP10. In abnormal situations, oncogenic activation of Ras signal transduction pathways leads to continuous upregulation of key elements of translational machinery. The Ras signal transduction pathways play a critical role in regulating mRNA translation and cellular transformation. On the other hand, tumor suppressor genes downregulate ribosomal and tRNA synthesis, and their inactivation results in uncontrolled production of these translational components. Heat shock proteins (chaperonins) are also a subgroup of oligomeric molecular chaperones. Chaperonins 10 and 60 can be found on the surface of various prokaryotic and eukaryotic cells, and release from cells. Secreted chaperonins can interact with a variety of cell types, including leukocytes, vascular endothelial and epithelial cells, and activate key cellular activities such as the synthesis of cytokines and adhesion proteins^[40-42].

We further observed that some receptor subunits (calmodulin) related to ion channels (potassium channel protein) were strongly upregulated (3.265-fold). Calmodulin (phosphorylase kinase delta) is a major cytoplasmic calcium receptor that performs multiple functions in cells including cytokinesis^[43]. Calmodulin is a small protein involved in calcium signaling and is able to bind to many different targets. The targets of calmodulin are a number of kinases, including myosin light chain kinase (MLCK), calmodulin-dependent kinase and phosphorylase kinase. The phosphorylase kinase holoenzyme (PhK) including alpha-beta, gamma and delta alters the interaction between its regulatory alpha and catalytic gamma subunits. The gamma subunit is also known to interact with the delta subunit, an endogenous molecule of calmodulin that mediates the activation of PhK by Ca (2+) ions^[44].

In conclusion, the down-regulated transcription factor Sox9 can influence cell differentiation and cell-specific gene expression. Down-regulation of heat shock protein 10 KD loses its inhibition of Ras, and attenuates the Ras GTPase signaling pathway, increases cell proliferation and inhibits cell apoptosis, which is a hallmark of aggressive malignant colorectal adenocarcinoma. Down-regulated Sox9 also decreases its binding to calmodulin, accumulates calmodulin as receptor-activated kinase and phosphorylase kinase due to the activation of PhK by Ca (2+) ions. Upregulated nuclear DNA-encoded mitochondria supply increases cancer cell proliferation energy.

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Loss of heterozygosity of Kras2 gene on 12p12-13 in Chinese colon carcinoma patients

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Abstract

AIM: To study the loss of heterozygosity (LOH) on 12p12-13 in Chinese colon carcinoma patients.

METHODS: DNA was extracted from 10 specimens of cancer tissue, 10 specimens of adjacent tissue and 10 specimens of normal tissue, respectively. LOH of Kras2 gene was analyzed by polymerase chain reaction (PCR) and denaturing polyacrylamide gel electrophoresis using 11 microsatellite markers on 12p-12-13.

RESULTS: LOH of Kras gene was detected at least on one marker of 12p-12-13 in 30% (3/10) of adjacent tissue specimens. The highest frequency of LOH was identified on D12S1034 in 28.57% (2/7) of adjacent tissue specimens. LOH was detected at least on one marker of 12p12-13 in 60% (6/10) of carcinoma tissue specimens, the most frequent LOH was found on D12S1034 and D12S1591 in 42.86% (3/7) of carcinoma tissue specimens. LOH was detected in 30% (3/10) of carcinoma tissue specimens, 30% (3/10) of adjacent tissue specimens, and no signal in 1% (1/0) carcinoma tissue specimen. The occurrence of LOH did not correlate with sex, age, tumor size and lymph node metastasis.

CONCLUSION: Genomic instability may occur on 12p-12-13 of Kras2 gene in the development and progression of colon carcinoma. The high LOH of Kras2 gene may directly influence the transcription and translation of wild type Kras2 gene.

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Key words: Colon carcinoma; Loss of heterozygosity; Kras2

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INTRODUCTION

Clinical and experimental studies have shown that point mutation of Kras2 gene plays an important role in the development and progression of tumors^[1-4]. However, it has been reported that wild type Kras2 gene has inhibitory effects on tumor growth and proliferation^[5]. Inactivation of cancer suppressor gene is a frequently encountered early event in the development of tumors, leading to loss of heterozygosity (LOH)^[6-10]. This study was to investigate the possible genetic variation of wild type Kras2 gene in the carcinogenesis of colon carcinoma by analyzing LOH in tumor and its adjacent tissues.

MATERIALS AND METHODS

Specimens

Ten specimens of primary carcinoma tissue, 10 specimens of its adjacent tissue and 10 specimens of normal tissue at the distal incision margin were taken respectively from patients with pathologically confirmed colon carcinoma before and after surgery at the Department of Surgery, General Hospital of the Chinese PLA from January to December 2003. The patients did not receive radiotherapy and chemotherapy before surgery.

DNA extraction from genome

Specimens of carcinoma and its adjacent tissues as well as normal tissue at the distal incision margin were suspended respectively in 50µL DNA lysate containing 50 mmol/L Tris-HCl, 1mmol/L EDTA, 0.1% Tween 20, 200 mg/L protease K, pH8.0, and digested overnight at 50°C. Protease K was denatured and inactivated at 95°C. Then the supernatant was centrifuged and stored at -20°C for use.

Primer design

Microsatellite-labeled primers on 12p12-13 were retrieved from the UniSTS database. The sequences of D12S89, D12S358, D12S310, D12S1606, D12S1596, D12S1592,

Table 1 Primer sequences on 12p12-13 microsatellite markers

| Markers | Primer sequences | Length (bp) |
|----------|---|-------------|
| D12S89 | D12S89-F:5' - ATTTGAGAGCAGCGTGTIT -3' D12S89-R:5' - CCATTATGGGGAGTAGGGGT -3' | 254-288 |
| D12s358 | D12s358-F:5' - GCCTTTGGGAAACTTTGG -3' D12s358-R:5' -TAAGCCCTTTTATTTTCCTAAC-3' | 238-270 |
| D12s310 | D12s310-F:5' - GAAAACTAATGCCCCTTAC -3' D12s310-R:5' - TTTAGATTCTCCAAATGCC -3' | 243-253 |
| D12S1606 | D12S1606-F:5' -ATGAGAGGCCAAAATTAAG -3' D12S1606-R:5' - CTACTGTGTGTCAGGGTCA -3' | 271-279 |
| D12s1596 | D12s1596-F:5' - TCATGTGGCTGGTAGAGAAG -3' D12s1596-R:5' - CGTGAAGCAGATTATCGTG -3' | 204-222 |
| D12s1617 | D12s1617-F:5' - AGCCTGAGGGCCACAT -3' D12s1617-R:5' - TGGGCAACTTGGATAAGAAACA -3' | 215-261 |
| D12s1592 | D12s1592-F:5' - GCTGAGTGTGGTGGCAC -3' D12s1592-R:5' - GAGACTATTCCAACAGTGTATTTTC -3' | 238-280 |
| D12S1057 | D12S1057-F:5' - AGAGACAGAAGCAGTAGGATGG -3' D12S1057-R:5' - GGTTCGACGTTTAAAGACTCG -3' | 227 |
| D12S1591 | D12S1591-F:5' - GCGCTTGTACTATGTTACATTT -3' D12S1591-R:5' - GAAGACGTTGGTGAATC -3' | 234-276 |
| D12S823 | D12S823-F:5' - GGGCAATAGAGTGAGATTCTG -3' D12S823-R:5' - CCTACCTCCTCCCATCC -3' | 119 |
| D12S1034 | D12S1034-F:5' - TTTTCAAAAAGTGTGACTGTGC -3' D12S1034-R:5' - CAGCCTAGTAAAAAATTTAAATTGG -3' | 280-302 |

D12S1617, D12S1057, D12S1591 are listed in Table 1.

Polymerase chain reaction (PCR)

One μ L 10xPCR buffer, 0.5 mmol/L magnesium ion, 0.2 mmol/L 4xDNTP, 0.4 μ mol/L upstream primer, 0.4 μ mol/L downstream primer, 1U Taq DNA polymerase/reaction and 0.5 μ L DNA template were added into 10 μ L PCR system at 94°C for 5 min. Fourteen PCR cycles of amplification were performed at 94°C for 30 s, at 60°C for 30 s, at 72°C for 30 s, followed by 16 cycles at 94°C for 30 s, at 55°C for 30 s, at 72°C for 30 s, and a final extension at 72°C for 5 min.

Denaturing polyacrylamide gel electrophoresis

PCR products (0.3 μ L) were added to the loading buffer containing GENESCAN-500 molecular weight as internal control and mixed with formamide, then denatured at 72°C for 2-3 min and electrophoresed by the AB1377 fluorescence sequencer. The standard sequencing PAGE 64-well denaturing polyacrylamide gel was used for electrophoresis. The electrophoresis was performed at the temperature higher than 42°C for 2.5-3 h. The electrophoresis channels were analyzed using GENESCAN version 3.1 Software. The types and intensity of fluorescence were collected for each channel, the size of amplified PCR products was determined using the molecular weight as internal control. Data of the type, intensity and molecular weight of the amplified microsatellite DNA fluorescence were obtained using GENOTYPE version 2.0 Software. The fluorescence intensity could indicate the amplified DNA within the linearity (relative fluorescence intensity was 200-300).

LOH analysis

Based on the principles of fluorescence labeling of primers, a fluorescence labeled primer group-matched

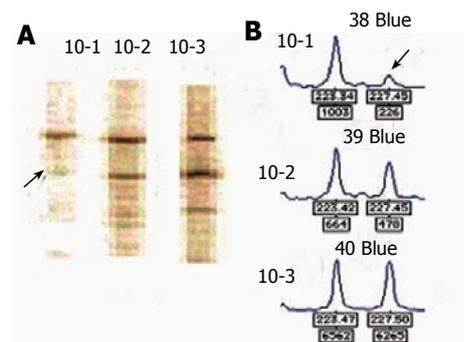


Figure 1 LOH on D12S1591 (A) and gene type (B) in carcinoma and normal tissues. The arrowhead shows the allele loss. 10-1: tumor, 10-2: adjacent tumor, 10-3: normal.

sequence was linked to the 5' end of a specific primer, so that the PCR products were labeled with the fluorescence group in the process of PCR. The fluorescence labeled PCR products were electrophoresed using the AB 1377 fluorescence sequencer. Data collected by the electrophoresis were analyzed using the GENESCAN and GENOTYPE Softwares to obtain the peak and size of the map. Gene typing was performed. The peak was found in 2 allelic gene segments and compared to the normal value of the adjacent channels. The allelic ratio was calculated according to the following formula: allelic ratio=peak ratio of carcinoma tissue/peak ratio of normal tissue. LOH was considered when the allelic ratio was higher than 1.5 or lower than 0.67. Microsatellite instability (MSI) was considered when no abnormal peak point was found in DNA of carcinoma tissue compared to normal tissue.

Statistical analysis

The correlation between LOH and clinical and pathological parameters was evaluated by chi-square test. All statistical analyses were carried out by SPSS 10.0. $P < 0.05$ was considered statistically significant.

RESULTS

Frequency of LOH in carcinoma adjacent tissue

No LOH was detected in 70 (7/10) of adjacent tissue specimens (1, 2, 4-6, 8, 9) on all markers. LOH was detected in 30% (3/10) of adjacent tissue specimens (3,7,10) at least on one marker, 28.5% (2/7) of adjacent tissue specimens on D12S1034, 25% (1/4) on D12S1617, 14.29% (1/7) on D12S1596, 12.5% (1/8) on D12S89, and 0% on other markers (Table 2 and Table 3, Figure 1).

Frequency of LOH in carcinoma tissue

No LOH was detected in 40% (4/10) of primary colon carcinoma tissue specimens (1, 4-6) on all markers. LOH

Table 2 Frequency of LOH on the 11 markers of 12p12-13 in colon carcinoma and its adjacent tissues

| Markers | Adjacent tumor tissue | | | | Tumor tissue | | | |
|----------|-----------------------|------------|----------------------|--------------------|--------------|------------|----------------------|--------------------|
| | LOH (n) | Signal (n) | Frequency of LOH (%) | Heterozygosity (%) | LOH (n) | Signal (n) | Frequency of LOH (%) | Heterozygosity (%) |
| D12S823 | 0 | 5 | 0 | 50 | 1 | 5 | 20 | 50 |
| D12S1034 | 2 | 7 | 28.57 | 70 | 3 | 7 | 42.88 | 70 |
| D12S1596 | 1 | 4 | 25 | 40 | 1 | 4 | 25 | 40 |
| D12S1591 | 1 | 7 | 14.29 | 70 | 3 | 7 | 42.88 | 70 |
| D12S358 | 0 | 8 | 0 | 80 | 2 | 8 | 25 | 80 |
| D12S310 | 0 | 3 | 0 | 30 | 1 | 3 | 33.33 | 30 |
| D12S1592 | 0 | 4 | 0 | 40 | 0 | 4 | 0 | 40 |
| D12S89 | 1 | 8 | 12.5 | 80 | 1 | 8 | 12.5 | 80 |
| D12S1617 | 1 | 7 | 14.29 | 70 | 1 | 7 | 14.29 | 70 |
| D12S1057 | 0 | 7 | 0 | 70 | 2 | 7 | 28.57 | 70 |
| D12S1606 | 0 | 6 | 0 | 60 | 1 | 6 | 16.67 | 60 |

Table 3 Distribution of LOH on 12p12-13 in microsatellite-labeled primers

| Biomarker | 1 | | 2 | | 3 | | 4 | | 5 | | 6 | | 7 | | 8 | | 9 | | 10 | |
|-----------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|----|---|
| | A | T | A | T | A | T | A | T | A | T | A | T | A | T | A | T | A | T | A | T |
| D12S823 | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ |
| D12S1034 | ○ | ○ | ○ | ○ | ● | ● | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ |
| D12S1596 | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ |
| D12S1591 | ○ | ○ | ○ | ● | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ |
| D12S358 | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ |
| D12S310 | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ |
| D12S1592 | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ |
| D12S89 | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ |
| D12S1617 | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ |
| D12S1057 | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ |
| D12S1606 | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ |

○: Normal; ○: No signal; ●: LOH; A: adjacent tissue T: cancer tissue

was detected in 60% (6/10) of colon carcinoma tissue specimens (2, 3, 7-10) at least on one marker, 42.86% (3/7) on D12S1034 and D12S1591, 33.33% (1/3) on D12S310, and 0% (0/4) on D12S1592 (Tables 2 and 3).

Correlation between LOH on 12p12-13 and clinicopathological parameters

Chi-square test was used to evaluate the correlation between LOH and clinicopathological parameters. The results showed that LOH did not correlate with age, sex, tumor size and lymph node metastasis (Table 4).

DISCUSSION

RASp21consisting of Hras1, Nras and Kras2, is a GTP-coupled protein and can transfer signals from cell surface into cells. Its normal expression is necessary to maintain the normal physiological activities of cells. Activated Ras proto-oncogenes, especially Kras2, play an important role in the carcinogenesis of human and rodent tumors. Mutations of Kras2 gene have been found in tumor tissues of human organs, including bladder^[11], breast^[12], rectum^[13], kidney^[14], liver^[15], lung^[16], ovary^[17], pancreas^[18], stomach^[19] and hematopoietic system^[20]. In general, about 30% cancers display ras gene mutations, while the highest mutation rate is found in colonic and pancreatic cancer^[21-25]. In samples

of mutated ras gene, most mutations occur in Kras2 gene. Mutation and activation of ras gene usually occur at codens 12 and 13 or 61, leading to the transformation of proto-oncogene to oncogene^[26]. This kind of activation can up-regulate the expression of ras/ErK signal channel in the absence of external stimuli and further increase the abnormality of associated signal channels, leading to malignant transformation of cells. Activated ras gene is usually considered as the dominant oncogene because of the existing expression of wild type ras and malignant transformation of activated ras^[27]. However, is still controversial the effect of the dominant gene-ras is still controversial since wild type ras has been found in human and mouse pulmonary adenocarcinomas^[28,29].

In vivo and *in vitro* experiments^[28] have shown that tumors are found more frequently in normal mice with 2 wild type Kras2 copies than in those with LOH of one wild type Kras2 copy after they are treated with 2 carcinogens. The occurrence of tumor is 50-fold higher in mice with LOH of one wild type Kras copy than in normal mice with 2 wild type Kras copies. The tumor in the former group of mice is poorly-differentiated adenocarcinoma, while the tumor in the later group of mice is adenoma. Zhang *et al*^[28] reported that wild type Kras2 gene can inhibit cell growth, formation of clones, and induce tumors in naked mice. In addition, LOH has

Table 4 Correlation between LOH on chromosome 12p12-13 and clinical pathologic factors

| Clinical character | LOH | | Total | Ratio (%) | P |
|-----------------------|-----|---|-------|-----------|----------|
| | + | - | | | |
| Tissue class | | | | | |
| Adjacent Tumor | 3 | 7 | 10 | 30 | 0.17753 |
| Tumor | 6 | 4 | 10 | 60 | |
| Age (yr) | | | | | |
| ≤50 | 4 | 3 | 7 | 70 | 0.77816 |
| >50 | 2 | 1 | 3 | 30 | |
| Sex | | | | | |
| Male | 5 | 2 | 7 | 70 | 0.25979 |
| Female | 1 | 2 | 3 | 30 | |
| Tumor size (cm) | | | | | |
| <5 | 3 | 1 | 4 | 40 | 0.42919 |
| ≥5 | 3 | 3 | 6 | 60 | |
| Lymph node metastasis | | | | | |
| Yes | 4 | 2 | 6 | 60 | 0.598161 |
| No | 2 | 2 | 4 | 40 | |

been found in pulmonary adenocarcinoma induced by various chemical carcinogens. Point mutation of *Kras2* gene is detected in 67-100% mice with LOH of wild type *Kras 2* gene. These important findings will certainly query the established carcinogenesis of dominant *Kras2* gene.

It was reported that the development and progression of colon carcinoma are a process involving multiple genes and factors, and characterized by its stages: normal mucosa → atypical hyperplasia including intestinal metaplasia → adenoma → adenocarcinoma^[30]. *Kras2* gene as a dominant oncogene due to its point mutation plays an important role in the progression of canceration, which is one of the reasons why the inhibitory effect of wild type *kras2* gene on cancer is concealed. Since cancer suppressor gene can be inactivated by deleting mutation, we studied LOH of *Kras 2* gene on 12p12-13 in primary colon carcinoma. LOH was detected in 30% (3/10) of adjacent tissue specimens at least on one marker, 28.5% (2/7) on D12S1034, suggesting that 12p12-13 is genomically insin precancerous cells, and that LOH on 12p12-13 may influence the expression of wild type *Kras2* gene in early colon carcinoma. LOH was detected in 60% colon carcinoma tissue specimens at least on one marker, which was higher than that in adjacent tissue, and 42.86% (3/7) on D12S1034, suggesting that the genomic instability of 12p12-13 exacerbates with the progression of carcinoma as demonstrated not only by the increasing number of allelic gene loss points but also by the increasing frequency of allelic gene loss at the same point. Such changes lead to a decrease of *Kras2* gene copies used for transformation. When another point mutation occurs in the process of carcinogenesis and produces dominant oncogene effects, the cancer suppressing function of wild type *Kras2* gene can be completely covered up.

In conclusion, *Kras2* gene can exert inhibitory effects on the proliferation of colon carcinoma cells. LOH on 12p12-13 does not correlate with the clinical and pathological parameters obtained from colon carcinoma.

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VIRAL HEPATITIS

Construction of eukaryotic expression plasmid containing HCV NS3 segment and protein expression in human HL-7702 hepatocytes

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Li JW, Li K, Jiang J, Xu XL, Huang ZQ. Construction of eukaryotic expression plasmid containing HCV NS3 segment and protein expression in human HL-7702 hepatocytes. *World J Gastroenterol* 2006; 12(7):1038-1042

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Abstract

AIM: To construct the eukaryotic expression plasmid containing HCV NS3 segment and to analyze the expression of NS3 protein in normal human hepatocyte HL-7702.

METHODS: We amplified HCV NS3 fragment from plasmid pBRTM/HCV 1-3011 containing the whole length of HCV genome, recombined it with expression vector pcDNA3.1(-) to form the eukaryotic expression vector pcDNA3.1(-)/NS3, and transfected human HL-7702 hepatocytes with the recombined plasmid by cationic polymers. The expressed HCV NS3 protein was detected and analyzed by immunohistochemical method and Western blot.

RESULTS: The amplified NS3 fragments had correct molecule weight and sequence. The successfully constructed eukaryotic expression plasmids were transfected to HL-7702 cells. The expressed NS3 proteins had correct molecular weight 70000.

CONCLUSION: Eukaryotic expression vector pcDNA3.1(-)/NS3 containing NS3 segment of HCV can be constructed, the sequence of NS3 fragments is consistent with the template. Normal human HL-7702 hepatocytes can efficiently express specific HCV NS3 protein *in vitro*.

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Keywords: HCV; Structural protein; Expression

INTRODUCTION

Hepatitis C virus (HCV) genome has only one open reading frame, which encodes for a precursor protein polymer containing 3000 amino acids. The protein products, including core, NS3, NS4B and NS5A, have an effect on hepatocellular carcinoma (HCC) induced by HCV. The non-structural protein 3 (NS3) segment plays a key role in replication of virus and is closely related with the growth of host cells. NS3 has attracted attention in many research fields of HCV. In this experiment, we constructed the eukaryotic expression vector containing HCV NS3 segment, which could express NS3 protein in normal human hepatocytes *in vitro*.

MATERIALS AND METHODS

Enzymes and reagents

PCR primers were synthesized by Shanghai Sangon Biological Engineering and Technology and Service (Shanghai, China). Restriction endonucleases *EcoRI* and *BamHI*, T4 DNA ligase, RNase A and Ex Taq DNA polymerase and markers DL 15 000 and DL 2000 were purchased from TaKaRa Biological Technology (Dalian, China). Protein radiant markers were purchased from Bioin Technology (Zhuhai, China). Plasmid purification kit and transmaster were purchased from BoLi Bioin Technology (Beijing, China). Anti-HCV NS3 monoclonal antibody was purchased from Biodesign(American). Donkey anti-sheep IgG-HRP was purchased from Kangchen Bio-tech (Shanghai, China). Immuno histochemical SP-HRP kit was purchased from Dingguo Biological Technology (Beijing, China). Western blot ECL kit was purchased from Bioin Technology (Zhuhai, China).

Vectors and experimental cells

Recombination plasmid pBRTM/HCV1-3011 containing the whole genome of HCV was donated by Professor

Rice (Washington, USA). Expression vector pcDNA3.1(-) containing CMV promoter ahead of multiple cloning sites, replication initiating site SV40ori as well as anti-ampicillin and anti-G418 sites, was donated by Doctor Shun Fenyong (College of Life Science and Technology, Jinan University). pMD 18-T simple vectors were purchased from TaKaRa Biological Technology (Dalian, China). *E. coli* JM109 was provided by Biochemistry Department Jinan University. HL-7702 cells were purchased from Shanghai Institute of Cell Biology (Shanghai, China).

Experimental groups

Non-transfected HL-7702, HL-7702 transfected with pcDNA3.1 (-)/NS3 and HL-7702 transfected with pcDNA3.1(-).

Construction of expression vector pcDNA3.1 (-)/NS3

Based on published sequences, the primers [5'-GAA TTC ATG GCG CCC ATC ACG GCG TAC GC-3' (upriver) and 5'-CTG GAC CTC CAG CAG TGC ATT CCT AGG-3' (downriver)] were used to amplify the whole sequence of HCV NS3 region. Thirty cycles of PCR were performed at 94 °C for 60 s, at 55 °C for 30 s, at 72 °C for 120 s, and at 72 °C for 10 min in 50 µl reaction mixture containing 0.3 µL (1 U/µL) Taq polymerase, 5 µL 10×PCR buffer, 1 µL dNTPs (20 mmol/L), 0.625 µL each primer (20 µmol/L), 1 µL DNA as template, and 41.45 µL distilled water. The PCR products were subjected to electrophoresis on 0.8% agarose gel for 60 min (voltage:80V), visualized by DNA viewer staining. The PCR products were connected to clone vector pMD 18-T after purification. Plasmid pMD 18-T/NS3 was transferred into *E. coli* with calcium chloride. The *E. coli* was cultured to amplify the plasmid. Successfully transferred *E. coli* was screened at 50 mg/L ampicillin. Then the constructed pMD 18-T/NS3 was recombined with expression vector pcDNA3.1(-)/NS3, thus the eukaryotic expression plasmid pcDNA3.1(-)/NS3 came into being.

Cell culture and growth curve

HL-7702 cells were cultured and passaged in RPMI 1640 medium supplemented with 10% fetal calf serum in an incubator containing 50 mL/L CO₂ at 37 °C. Cells were digested by 0.25% trypsin and seeded (2.5×10⁴ cells per well) in 48-well culture plates. Cells were digested and counted at an interval of 24h with the average number of cells in 3 wells calculated per time. The detection was continued for 8 d. Then the population doubling time was calculated and growth curve of the cells was plotted.

Immunohistochemical staining

We used S-P method to detect the expression of HCV NS3 protein in HL-7702 cells transfected with plasmid pcDNA3.1 (-)/NS3. Cells transfected with and without plasmid pcDNA3.1 (-) served as negative and blank control groups. Cells were cultured in 25-flasks till 85% convergence. The cells were washed three times with pre-cooled PBS before harvested. Then 1 milliliter lysis reagent containing 50 mmol/L TrisHCl (pH8.0), 150 mmol/L NaCl, 0.02% sodium azide, 0.1% SDS, 100 mg/L PMSF

(dissolved in isopropyl alcohol, 1 mg/L aprotinin, 1% Nonidet P-40, 0.5% sodium deoxycholate) was added and incubated at 4 °C for 20 min. Then the lysis reagent mixed with broken cells was harvested and centrifuged at 12 000 r/min for 5 min. Twenty microliters of supernatant protein was resolved by SDS-8% polyacrylamide gel electrophoresis. After electrophoresis, the proteins were transferred onto a PVDF membrane, blocked in 5% skimmed milk for 1h, probed with monoclonal antibody to HCV NS3(1:1000) overnight at 4 °C. After washed three times with TBS containing 0.1% Tween 20, the membrane was treated with horseradish peroxidase-conjugated donkey anti-goat antibody for 3 h. Protein binding was detected by chemiluminescence reagent (ECL). Then the bands on X-film were assayed by densitometric scanning.

RESULTS

Construction of expression vector pcDNA3.1(-)/NS3

The PCR product of NS3 had correct molecule weight. Recycled PCR fragments were connected to T vector, *E. coli* was used to amplify the recombined vector pMD 18-T/NS3. Plasmid extracted from the positive clones could be digested by *EcoRI* and *BamHI* to two fragments (3000bp and 1900kb) as expected. DNA sequence analysis confirmed that the inserted fragment was a HCV NS3 segment containing 1893 nucleotides with right direction and reading frame. Initial codon, terminal codon and restriction sites of *EcoRI*, *BamHI* were successfully added to the ends. Vector pMD 18-T/NS3 was successfully recombined with plasmid pcDNA3.1(-). The length of the recombined vector pcDNA3.1(-)/NS3 was 7 300bp. After digested by *EcoRI* and *BamHI*, pcDNA3.1(-)/NS3 was cloven into two fragments (5 400 bp and 1900 kb) as expected (Figure 1A-1C).

The incubation condition of HL-7702 cells and the cell growth curve were obtained (Figure 2 A, 2B)

Immunohistochemical analysis showed that the blank control group was negative, pcDNA3.1 (-)/NS3 transfection group was positive for HCV NS3 protein in cytoplasm. SDS-PAGE analysis showed that among the separated protein bands (distilled from cytoplasm), a band was found at molecular weight 70 000 as expected (Figure 3A-3D)

DISCUSSION

Hepatitis C virus (HCV), the major etiological agent of non-A non-B hepatitis identified by the end of 1980s, has infected more than 170 million persons worldwide and 85% of them could develop chronic hepatitis, liver function failure or hepatocellular carcinoma. Among all the gene segments of HCV, NS3 segment has many important physiological functions. Genetic variability of NS3 protein lies in different kinds of HCV-infected patients^[2]. Nowadays little has been known about the nonstructural protein of HCV. NS3 protein contains many enzymes such as helicase, NTPase and serine protease, which can degrade the nonstructural segments of HCV polyprotein^[3-5]. HCV core, NS3, NS5A and NS5B

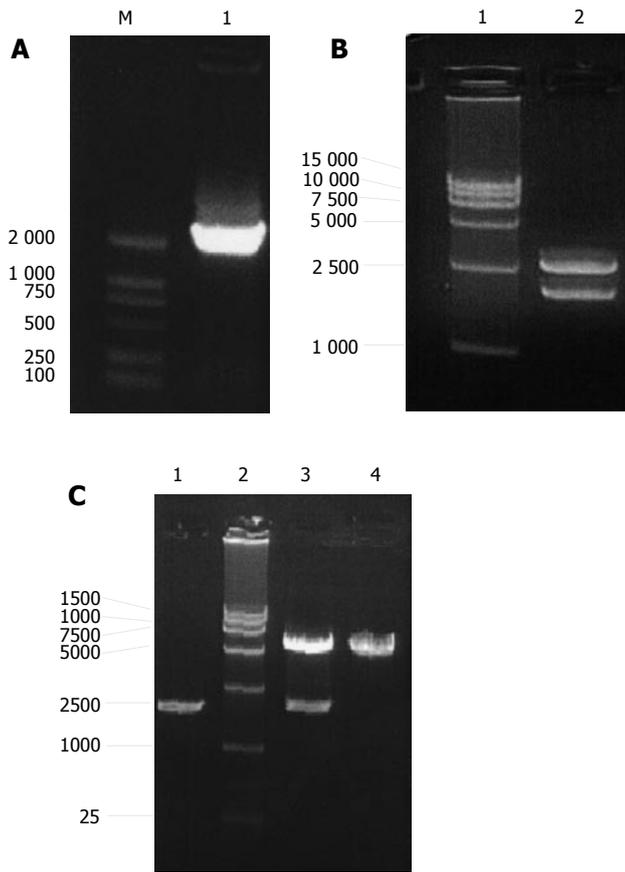


Figure 1 PCR results of NS3 gene(A), pMD 18-T/NS3 (B) and pcDNA3.1(-)/NS3 (C) digested with Eco and BamHI.

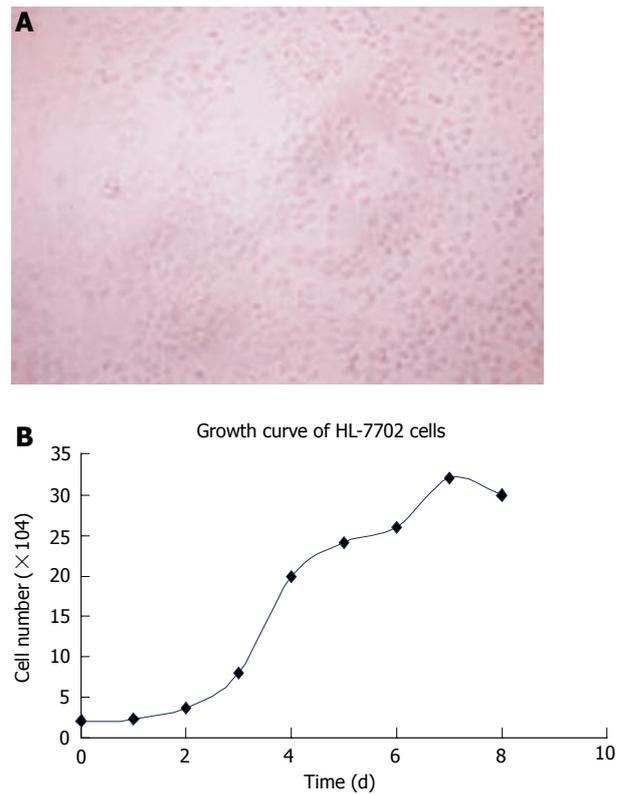


Figure 2 Convergence (A) and growth curve (B) of HL-7702 cells.

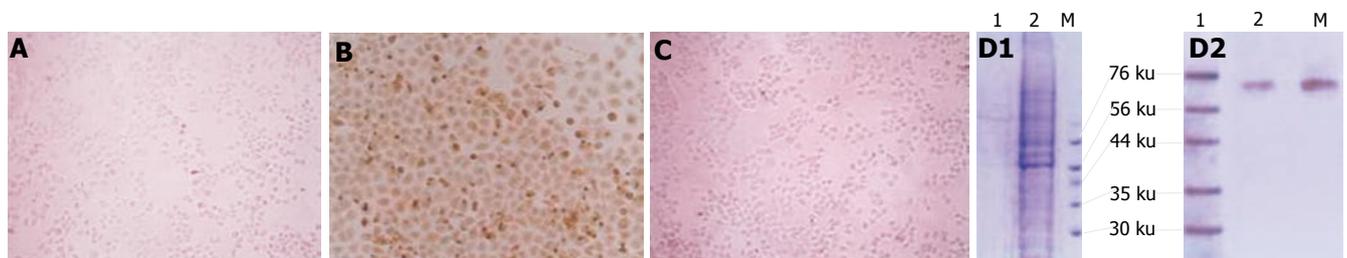


Figure 3 Immunohistochemical staining of NS3 protein expressed in non-transfected (A), transfected with pcDNA3.1(-)/NS3 (B), pcDNA3.1(-) (C) HL-7702 cells SDS-PAGE and Western blot as well as of NS3 protein expression in HL-7702 cells (D1-D2).

proteins can modulate cell proliferation independently by expressing p53^[6]. Chronic hepatitis C induced by HCV could do great harm to human body, yet little has been known about the pathogenetic mechanism. HCV can impair the innate immunity system. It was reported that interaction between HCV NS3 protein and host TBK1 protein leads to inhibition of cellular antiviral responses^[7]. Conserved C-terminal threonine of HCV NS3 can regulate autoproteolysis^[8]. NS3 protein can down-regulate activities of protease peptide which could interfere with the activity of viral antigens, which may protect HCV from host immune surveillance, leading to persistent viral infection^[9]. Besides, NS3/4A-mediated cleavage of TIR domain-containing adaptor protein potentially limits expression of multiple host defense genes, promoting

persistent infections with this medically important virus^[10]. Dolganiuc *et al*^[11] showed that core protein and nonstructural protein 3 of HCV can induce production of IL-10 and mediate immunosuppression of against HCV. Some HCV proteins, such as core protein, NS3 and NS5A, not only regulate cell growth and interact with other cellular proteins, but also participate in programmed cell death under certain circumstances^[12]. HCV can interfere with JAK/STAT signal pathway, leading to IFN production by down-regulating the expression of STAT1^[13]. Additionally, NS3 can bind to a host protein (Sm-D) which interferes with the formation of small nuclear ribonuclear protein (snRNP) complexes, so that occurrence of auto immune disease can be reduced^[14]. It was reported that nonstructural protein 3 of HCV

can inhibit dendritic cell differentiation by inducing pro- and anti-inflammatory cytokines^[15]. Core, NS3, NS5A, NS5B proteins of hepatitis C virus can induce apoptosis of mature dendritic cells^[16] and inhibit cellular immune responses through impairment of maturation of dendritic cells^[17]. Dolganiuc *et al.*^[18] found that HCV core and NS3 proteins could activate monocytes. NS3 protein can activate innate immunity to recognize virus more efficiently through inducing inflammation^[18]. Studies indicate that persistent HCV infection is closely related with hepatocellular carcinoma, replication and protein expression of HCV have been observed in HCC tissues^[19-21], suggesting that interaction between HCV gene segments and expressed protein plays a key role in malignant transformation of hepatocytes^[22]. Feng *et al.*^[23] showed that HCV NS3 N-terminal peptide may up-regulate the activation of MAPK, but not affect the expression of MAPK in normal liver cells. Additionally, Sakamuro *et al.*^[24] reported that NS3 gene-expressed protein in NIH3T3 cells and mouse fibroblasts can be transfected with NS3 gene segment, which induces tumors in nude mice. NS3 protein can inhibit actinomycin D-induced apoptosis^[25] and inhibit phosphorylation mediated by cAMP-dependent protein kinase^[26]. Activation of c-Jun NH2-terminal kinase (JNK) signaling pathway is essential for the stimulation of HCV non-structural protein 3 (NS3)-mediated cell growth^[27]. It has been shown that cells consistently expressing low concentration of NS3 protein is prone to apoptosis induced by the Fas molecule^[28].

In this experiment, we used pcDNA3.1(-) vector to construct the eukaryotic expression vector pcDNA3.1(-)/NS3. DNA sequence analysis revealed that the inserted NS3 gene had correct reading frame and length(1893nt). Initial and terminal codons were successfully inserted into the ends of NS3 segment, which assured the correct expression of NS3 protein. We chose adult human HL-7702 hepatocytes as the host cells. The cells originate from embryonic liver, and were continuously cultivated *in vitro*. HL-7702 cells are slightly larger than normal human hepatocytes *in vitro*. The nuclei/cytoplasm ratio is high and cells are joined to each other tightly^[29]. We transfected the constructed eukaryotic plasmid pcDNA3.1(-)/NS3 to HL-7702 cells, and detected the expressed protein by immunohistochemical staining. NS3 protein was expressed in the transfected cells, but not in blank cells and cells transfected with pcDNA3.1 (-). Western blot analysis showed that the expressed NS3 protein had correct molecular weight (70 000). The successful construction of eukaryotic plasmid pcDNA3.1 (-)/NS3 and correct expression of HCV NS3 protein in HL-7702 cells may contribute to the establishment of a system that can produce NS3 protein. These results may help further investigation of how NS3 protein affects immunity against virus.

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Screening and identification of interacting proteins with hepatitis B virus core protein in leukocytes and cloning of new gene C1

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Abstract

AIM: To investigate the biological function of HBcAg in pathogenesis of HBV replication in peripheral blood mononuclear cells (PBMCs).

METHODS: HBcAg region was amplified by polymerase chain reaction (PCR) and HBV HBcAg bait plasmid pGBKT7-HBcAg was constructed by routine molecular biological methods. Then the recombinant plasmid DNA was transformed into yeast AH109. After the HBV core protein was expressed in AH109 yeast strains (Western blot analysis), yeast-two hybrid screening was performed by mating AH109 with Y187 containing leukocyte cDNA library plasmid. Diploid yeast cells were plated on synthetic dropout nutrient medium (SD/-Trp-Leu-His-Ade) (QDO) and synthetic dropout nutrient medium (SD/-Trp-Leu-His-Ade) (TDO). The second screening was performed with the LacZ report gene (yeast cells were grown in QDO medium containing X- α -gal). The interaction between HBV core protein and the protein obtained from positive colonies was further confirmed by repeating yeast-two hybrid. After plasmid DNA was extracted from blue colonies and sequenced, the results were analyzed by bioinformatic methods.

RESULTS: Eighteen colonies were obtained and sequenced, including hypermethylated in cancer 2 (3 clones), eukaryotic translation elongation factor 2 (2 clones), acetyl-coenzyme A synthetase 3 (1 clone), DNA polymerase gamma (1 clone), putative translation initiation factor (1 clone), chemokine (C-C motif) receptor 5 (1 clone), mitochondrial ribosomal protein

L41 (1 clone), kyo binding protein genes (1 clone), RanBPM (1 clone), HBeAg-binding protein 3 (1 clone), programmed cell death 2 (1 clone). Four new genes with unknown function were identified.

CONCLUSION: Successful cloning of genes of HBV core protein interacting proteins in leukocytes may provide some new clues for studying the biological functions of HBV core protein.

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Key words: Hepatitis B virus; Core protein; Yeast two-hybrid system

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<http://www.wjgnet.com/1007-9327/12/1043.asp>

INTRODUCTION

Hepatitis B virus (HBV) infection is a global challenge with 350 million chronic carriers world wide. Individuals with chronic HBV infection have a high risk of developing liver cirrhosis and primary hepatocellular carcinoma^[1, 2]. HBV is a partially double-stranded DNA with compact circular genome of about 3200 base pairs (bp) containing four overlapping open reading frames P, preS/S, preC/C and X. C region coding for the HBV core antigen (HBcAg)^[3, 4] is a 21-22 kD protein that self-assembles to form the subviral 30-32 nm nucleocapsid particles packing the viral polymerase and pregenomic RNA during HBV replication. HBcAg can prevent the degradation of mRNA by RNA-enzyme and plays a very important role in assembling pregenome and synthesizing genome^[5-7].

Some researchers have detected HBV nucleic acids including replicating intermediates, covalently closed circular DNA(cccDNA), viral RNA transcripts and DNA sequences integrated with the celler genome in PBMCs from patients with active chronic hepatitis B (CHB)^[8-13]. It was reported that patients with hepatocellular carcinoma (HCC) display a higher prevalence of HBV in PBMCs

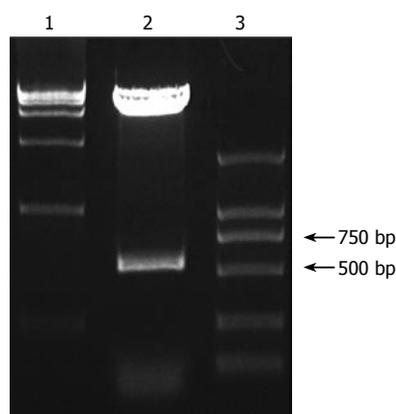


Figure 1 pGBKT7- HbcAg cut by *EcoRI* and *PstI* on 0.9% agarose/EtBr gel. Lanes 1 and 3: Marker; lane 2: HbcAg fragment cut by *EcoRI* and *PstI*.

than asymptomatic carriers and CHB patients or liver cirrhosis patients, although the detection rate of viral replication is low in HCC patients^[14]. Viral load in serum is thus not always associated with the prevalence of HBV DNA in PBMCs, and cccDNA may exist even in patients with undetectable HBV DNA from serum by PCR. The pathogenesis of replication and integration of HBV in PBMCs is unclear. However, it may contribute to viral escape from the immune system and pathogenesis of lympho-proliferative disorders^[15].

In order to further reveal the biological role of HbcAg in pathogenesis of replication and integration of HBV in PBMCs, we tried to identify its associated proteins in the present study by the GAL4-based yeast two-hybrid system using HbcAg as bait to screen the human leukocyte cDNA library.

MATERIALS AND METHODS

Yeast strains, chemical agents and cultural media

Bacterial yeast strains and plasmids for yeast-two hybrid experiments were obtained from Clontech Co., USA. Yeast strain AH109 (MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2: GAL1UAS-GAL1TATA-HIS3, GAL2UAS-GAL2TATA-ADE2 URA3: MEL1 TATA-lacZ MEL1) containing pGBKT7-53, coding for DNA-BD/mouse p53 fusing protein and AH109 was used for cloning of bait plasmids. Yeast strain Y187 (MATa ura3-52, his3-200, Ade2-101, trp1-901, leu2-3, 112, gal4Δ, gal80Δ, met-, URA3::GAL1UAS-GAL1TATA-lacZ MEL1) containing pTD1-1, in which pACT2 coding for AD/SV40 large T antigen fusing protein and Y187 was used for cloning of library plasmids. Bacterial strain DH5a was used for cloning of each shuttle plasmid. TEMED was from Boehringer Mannheim Co., USA. Tryptone and yeast extracts were from OXOID Co., USA. X-α-Gal and cultural media were from Clontech Co., USA. The primers of PCR were from Boya Co., Shanghai, China.

Construction of "bait" plasmid

Plasmid pGBKT7-HbcAg (Figure 1) containing full-length HBV core gene was constructed by inserting HBV core gene in-frame into *EcoRI*/*PstI* site, which could directly

express DNA binding domain, *c-myc* and core fusion protein. The construct was verified by restriction digestion and sequencing (Figure 1). The plasmid was transformed into yeast strain AH109 by the lithium acetate method^[16]. Transformed AH109 was cultured on quadruple dropout media to exclude its auto-activity.

Expression of HBV core protein

Denatured cell extracts were subjected to electrophoresis using 4-12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes as previously described^[17]. The membranes were blocked with 5% nonfat dry milk for 1 h and then incubated with monoclonal anti-*c-myc* antibody (Santa Cruz Biotechnology INC, Santa Cruz, CA, USA) for 2h followed by HRP-conjugated secondary antibody for another 1h prior to detection of antibody reactive proteins.

Yeast-two hybrid screening

The screening of leukocyte cDNA library was performed as previously described^[18,19]. One large (2-3mm) fresh (<2 mo old) colony of AH109 [bait] was inoculated into 50 mL of SD/-Trp and incubated at 30 °C overnight (16-24 h) with shaking at 250-270 r/min. Then the cells were spun down by centrifuging the entire 50-mL culture at 1000r/min for 5 min and supernatant decanting the cell pellet was re-suspended in the residual liquid by vortexing. The entire AH109 [bait] culture (beyond 1:10⁹) and 1mL library (beyond 1:10⁹) were combined and cultured in a 2L sterile flask and 45 mL of 2 X YPDA/Kan was added and swirled gently. After 20 h mating, the cells were spun down, re-suspended and spread on 25 large (150 mm) plates containing 200 ml of SD/-Ade/-His/-Leu/-Trp (QDO) or 200 ml of SD/-His/-Leu/-Trp (TDO). After 6-18 d, the yeast colonies larger 3 mm in diameter were transferred onto the plates containing X-α-Gal to check expression of the MEL1 reporter gene (blue colonies).

Isolation of plasmid DNA from putatively positive yeast clones and bioinformatic analysis

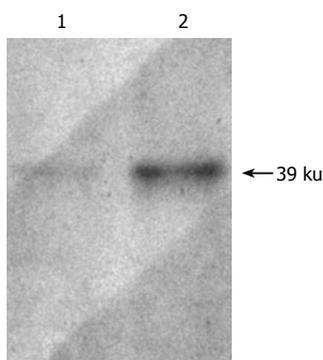
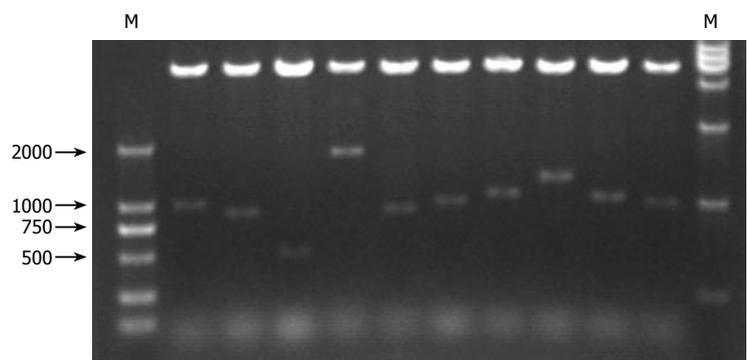
Yeast plasmid was isolated using the lyticase method (Clontech Co., USA). The plasmid was transformed into *E. coli* strain DH5α. Transformants were plated on ampicillin LB selection media (SOB) and plasmids were isolated from *E. coli*. Restriction digestion and sequencing analysis were carried out. After the positive colonies were sequenced, the sequence was blasted with GenBank to analogize the function of the genes^[20,21].

New genes cloned

The coding sequence of new gene was obtained from the cDNA library of proteins binding to core protein of hepatitis B virus by bioinformatic methods, named C1. Standard PCR cloning technique was used to amplify C1 gene. Cytoplasmic RNA of Jurkat cells was isolated and used for RT-PCR. The primer sequences of the sense and antisense are 5'-GAA TTC ATG GAG GAG GTC ATA C-3' and 5'-GGA TCC TTA CCA GGG ACA CAG T-3', respectively. The PCR products were cloned with pGEM-T vector (Promega Co., USA). Primary structure of the insert was confirmed by direct sequencing.

Table 1 Sequence analysis of 18 clones interacted with the HBcAg protein

| Known genes | Clone number | Homology (%) |
|--|--------------|--------------|
| Homo sapiens hypermethylated in cancer 2 (HIC2) | 3 | 98-100 |
| Homo sapiens eukaryotic translation elongation factor 2 (EEF2) | 2 | 100 |
| Homo sapiens acetyl-Coenzyme A synthetase 3 | 1 | 99 |
| Homo sapiens DNA polymerase gamma (POLG), nuclear gene encoding mitochondrial protein | 1 | 99 |
| Homo sapiens putative translation initiation factor(SUI1) | 1 | 99 |
| Homo sapiens chemokine (C-C motif) receptor 5 | 1 | 100 |
| Homo sapiens mitochondrial ribosomal protein L41 (MRPL41), nuclear gene encoding mitochondrial protein | 1 | 100 |
| Homo sapiens Kyot binding protein | 1 | 99 |
| Homo sapiens mRNA for RanBPM | 1 | 99 |
| Homo sapiens HBeAg-binding protein 3 (HBEBP3) | 1 | 99 |
| Homo sapiens programmed cell death 2 (PDCD2) | 1 | 100 |
| New genes with unknown function | 4 | 97-100 |

**Figure 2** Western blotting shows the expression of HBcAg in yeast. Lane 1 is negative control and lane 2 is HBV core protein.**Figure 3** Screening colonies cut by Bgl II on 0.9% agarose/EtBr gel

Interaction between HBV core protein and new gene C1-translated protein in yeast

PGADT7-C1 plasmid was constructed and transformed into yeast strain Y187 using the lithium acetate method after sequencing analysis. Mating experiments were carried out by mating yeast strain AH109 containing pGBKT7-HBcAg plasmid with Y187 containing pGADT7-C1. The diploid yeasts were plated on media lacking leucine, tryptophan, histidine and adenine^[22].

RESULTS

Construction of "bait" plasmid pGBKT7-HBcAg and expression of "bait" fusion protein

The full-length HBcAg gene was cloned into the yeast two-hybrid BD vector at the *EcoR I* and *Pst I* sites. The construct was verified by restriction digestion and sequencing (Figure 1).

Western blotting was performed to confirm the expression of the fusion protein using *c-myc* monoclonal antibody. Yeast strain AH109 transformed with pGBKT7-HBcAg could stably express the fusion protein at a high level but could only grow on SD/-Trp medium (Figure 2).

Yeast-two hybrid cloning of protein interacting with HBcAg

HBV-core protein was used as the bait for screening

human yeast-two hybrid leukocyte cDNA library. The clones growing in the absence of tryptophan, leucine, histidine and adenine were processed for β -galactosidase assay, and blue colonies were picked. As the plasmid pACT2 contains two sites for *Bgl II* enzyme digestion, we could get the screening gene fragment of leukocyte library by *Bgl II* enzyme digestion (Figure 3). The different size of DNA segments showed that the screening colonies were positive clones.

Sequence analysis of positive clones

Nucleotide sequences of the clones from the cDNA library were analyzed, the full-length sequences were obtained with Vector NTI 6 and BLAST database homology search (<http://www.ncbi.nlm.nih.gov/>) (Table 1).

Confirmation of new gene expression by RT-PCR

The PCR products were cloned with pGEM-T vector. The direct sequencing showed the ORF of C1 (Figure 4, Figure 5). The full length of the coding rank contained 366 nucleotides, and the coding product consisted of 121 amino acid residues (GenBank accession number: AY555145).

Mating experiments

To confirm the true protein-protein interaction (protein

ATG GAG GAG GTC ATA CAA GCA GGC CTG GCC
M E E V I Q A G L A
CAG TGG TCC AGA CAG AAG GGC CTG GCC TTG
Q W S R Q K G L A L
CCC TGG GAC AGA ACC AGA GGC CAT CCT GAT
P W D R T R G H P D
GTT CCC TGG AGA AAT CTC ACC TCC TCA CCC
V P W R N L T S S P
ACC AGG CCA TTG GCC CAG CCT GCC GGA AGC
T R P L A Q P A G S
TGC ATG CCA GCG GAG CCC AGC CCT GCT GCC
C M P A E P S P A A
CAC TAC CAC CAG CTC CAT GTG CAC CTC CAG
H Y H Q L H V H L Q
CTC TTG CCC TCT GAC TTG TCT GAG CGT CCC
L L P S D L S E R P
GGG CTT AGG CTG GCC CCA CTG GCC CTG GTG
G L R L A P L A L V
GAG GTG GGG ATG ACT CTC CCA GTG CCA CAG
E V G M T L P V P Q
AAA CTG GCT CCT GGG CGG CAA CTG TGT CCC
T P L P H V T Q Q Q
ACT CCC CTC CCC CAC GTA ACC CAG CAA CAG
K L A P G R Q L C P
TGG TAA
W *

Figure 4 The nucleotide sequence of C1 gene and relevant amino acid sequences.

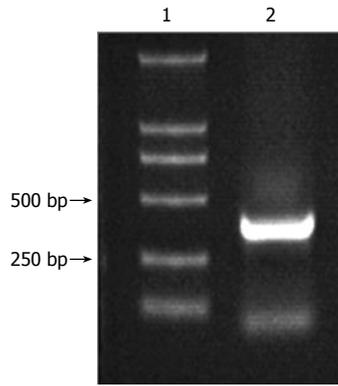


Figure 5 C1 DNA fragment amplified by RT-PCR. Lane 1 is Marker and lane 2 is a 366 bp fragment of C1, amplified by RT-PCR.

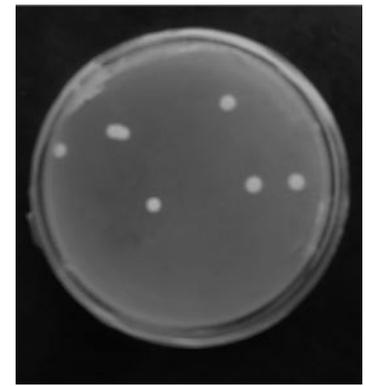


Figure 6 Positive clones interactive with the HBcAg protein grew on media lacking leucine, tryptophan, histidine and adenine.

of HBcAg and new gene C1-translated protein) in yeast, mating experiments were carried out by mating yeast strain AH109 containing the plasmid of pGBKT7-HBcAg with Y187 containing pGADT7-C1. The diploid yeast cells were plated on QDO (Figure 6).

DISCUSSION

The interaction of proteins is the basement of life, and gene shows its function only by this way. The yeast-two hybrid system relies on the modulating properties of eukaryotic site-specific transcriptional activators for the detection and analysis of protein-protein, protein-DNA and protein-RNA interactions. This onslaught of genomic information calls for the development of efficient experimental strategies to gain insight into the organization and function of the encoded proteins. The yeast-two hybrid system can detect protein-protein interactions. Yeast-two hybrid system 3 is based on the originally designed system^[23-25]. In this system, the promoters controlling HIS3, ADE2, and MEL1 expression in AH109 have significantly fewer false positives and the simple mating protocol significantly reduces the labor and time involved in performing a yeast-two hybrid library screening and improves the chances of finding rare protein-protein interactions and leads to more reproducible results^[26,27].

HBcAg has a conservative three-dimensional structure, 21 kD. Its C-terminal 150-185 aa is rich in arginine region. One function of the arginine-rich region is to provide interactions between core protein and RNA pregenome. Packaging the viral pregenomic RNA into HBV nucleocapsid (core particle) is a crucial function of the core protein^[28]. The C-terminal is in the region of interaction between HBcAg and RNA/DNA and is phosphorylated by cell kinase. Phosphorylation is very important for the synthesis and replication of DNA. Phosphorylation of the core protein is an important step in viral replication cycle necessary for transport of the viral genome to the nuclei^[29, 30]. HBV capsid or core protein binds to nucleic

acid through a carboxy-terminal protamine region containing nucleic acid-binding motifs organized into four repeats (I to MV). Core proteins contain nuclear localization signals (NLS) in their COOH termini and mediate HBcAg into nuclei^[29, 31]. HBcAg showing a high immunogenicity can directly activate B lymphocytes by binding to the region of FR1-CDR1 of B cells^[7].

HBV has been found in numerous extrahepatic cells including PBMCs. However, their role in HBV infection and viral persistence remains unclear. The presence of HBV-DNA and HBV-RNA in PBMCs has been demonstrated by Pontisso *et al*^[32]. Polymerase chain reaction (PCR) showed that HBV infection of PBMCs is a common event in acute and chronic hepatitis B^[33]. Since HBV is cytopathogenic, viral proteins elicit a modulating influence on the gene expression of infected immunocompetent cells^[34].

In our study, the bait plasmid pGBKT7-HBV core was constructed and the gene of HBcAg was expressed in yeast AH109. We identified 18 colonies coding for 11 different proteins. These genes encode proteins involving in cell signal transduction, cancer development, and immunity pathogenesis. The two families of chemokines were distinguished on the arrangement of the first two of four conserved cysteines. The α -chemokine is characterized by two cysteine amino acids separated by another amino acid (C-X-C motif), while the β -chemokine has two adjacent cysteine residues (C-C motif). The majority of β -chemokines are monocyte chemoattractants. Chemokine receptors are members of the seven receptors of transmembrane or serpentine family^[35, 36]. The receptors are G-protein-linked and transducer signals via phospholipase C. This subsequently leads to a rise in intracellular calcium concentration and activation of protein kinase C. Chemokine (C-C motif) receptor 5 contains monocytes, but it was reported that CCR5 can be expressed on Th1 and Th2 cells selectively^[37]. HIV can infect the Th1 cells by synergistic action of CCR5, and cause fusion of the infected cells and HIV diffusion^[38].

A candidate tumor suppressor gene hypermethylated in cancer (HIC-1) has been identified because of its association with a "CpG island" at 17p13.3 that is hypermethylated and transcriptionally inactivated in several common types of human cancers^[39]. HIC encodes a typical nuclear BTB/POZ protein with five Kruppel-like C₂H₂ zinc finger motifs in its C-terminal and a BTB/POZ domain at its N-terminal. BTB/POZ protein found in developmentally regulated transcription factors and actin-binding proteins is associated with C₂H₂ zinc finger motifs in proteins involved in transcriptional regulation through chromatin modeling^[40, 41]. Kyot binding protein genes code for KBP1, KBP2, KBP3. KyoT2 binds to KBP in LIM, which is rich in amino-3-mercaptopyronic acids and can bind to ZN and protein kinase C^[42]. RanBPM cDNA codes for a 90 kD protein. RanBPM is a peripheral membrane protein. Integrins and RanBPM interact *in vitro* and *in vivo* and co-localize on the cell membrane. RanBPM may constitute a molecular scaffold that contributes to coupling. The leukocyte integrin lymphocyte function-associated antigen 1 (LFA-1) and other integrins are found to intracellular signaling pathways. Ran is a Ras-like GTPase, which can transport nucleoplasm, assemble microtubules and form nuclear membrane. RanBPM exists in the center of cells as a receptor of Ran related with transport nucleoplasm^[43-45].

In our study, 4 new genes with unknown function were identified. One of these new genes was named C1 with no homology sequence in GenBank. By electron splicing, the opening reading code framework and full-length of this gene were concluded. The gene can be transcribed from the mRNA of Jurkat cells by RT-PCR. The PCR product was analyzed by T-A sequence. The full length of the coding rank contained 366 nucleotides, and the coding product consisted of 121 amino acid residues. The pGADT7-C1 plasmid was constructed and transformed into the yeast strain Y187. The Y187 and AH109 were mated, the result showed that the two proteins could bind to each other.

In conclusion, HBcAg can bind to proteins in leukocytes.

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PI 3-kinase pathway is responsible for antiapoptotic effects of atrial natriuretic peptide in rat liver transplantation

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an event triggering antiapoptotic signaling cascade in ischemic liver.

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Abstract

AIM: To investigate the *in vivo* effect of atrial natriuretic peptide (ANP) and its signaling pathway during orthotopic rat liver transplantation.

METHODS: Rats were infused with NaCl, ANP (5 µg/kg), wortmannin (WM, 16 µg/kg), or a combination of both for 20 min. Livers were stored in UW solution (4 °C) for 24 h, transplanted and reperfused. Apoptosis was examined by caspase-3 activity and TUNEL staining. Phosphorylation of Akt and Bad was visualized by Western blotting and phospho-Akt-localization by confocal microscopy.

RESULTS: ANP-pretreatment decreased caspase-3 activity and TUNEL-positive cells after cold ischemia, indicating antiapoptotic effects of ANP *in vivo*. The antiapoptotic signaling of ANP was most likely caused by phosphorylation of Akt and Bad, since pretreatment with PI 3-kinase inhibitor WM abrogated the ANP-induced reduction of caspase-3 activity. Interestingly, analysis of liver tissue by confocal microscopy showed translocation of phosphorylated Akt to the plasma membrane of hepatocytes evoked by ANP.

CONCLUSION: ANP activates the PI-3-kinase pathway in the liver *in vivo* leading to phosphorylation of Bad,

INTRODUCTION

Ischemia reperfusion injury (IRI) is responsible for primary liver dysfunction and failure after transplantation or liver resection^[1,2]. Therefore, reduction of IRI is of high clinical interest. In order to develop preventive strategies, knowledge of the mechanisms leading to cell death after IR is pivotal. The mode of cell death after IR is originally considered to be necrotic, but there is increasing evidence that apoptosis plays a role^[3-5]. Apoptosis is a highly regulated process resulting in nuclear fragmentation and condensation as well as disintegration of cells into apoptotic bodies without release of cell contents. Subsequently, tissue macrophages such as Kupffer cells phagocytose the apoptotic bodies. In contrast to apoptosis, necrosis is a passive process characterized by cell lysis. Usually, necrosis of cells causes a local inflammation. However, it has been recognized that apoptotic cells also induce inflammatory response by triggering neutrophil accumulation into the liver^[6]. Thus antiapoptotic strategies convey protection against hepatic IRI. Apoptosis occurs during the early phase of reperfusion after liver ischemia and after transplantation^[3,5,7]. However, the extent to which it occurs and most importantly its relevance in IRI is still a matter of debate, which might be due to different experimental models for liver IRI^[5,7].

Atrial natriuretic peptide (ANP) belongs to the natriuretic peptide family including a number of peptides possessing vasodilating, hypotensive and natriuretic activities^[8]. In addition to this cardiovascular profile there

is ample evidence that ANP exerts cytoprotective actions in various cells and organs besides the liver^[9-12].

In the model of isolated perfused rat liver we observed a rather low amount of apoptotic cells after cold ischemia compared to other reports^[5]. We could further show that pretreatment of liver with ANP could lead to a significant reduction of hepatocyte apoptosis and necrosis^[5]. ANP-treatment has also been shown to be beneficial in isolated rat hepatocytes exposed to hypoxia^[13].

This study was to examine the effect of ANP-preconditioning in an *in vivo* model of liver IRI rat orthotopic liver transplantation (IRI) and the mechanism of cell protection.

MATERIALS AND METHODS

Materials

Rat ANP, wortmannin and protein kinase A assay kit were purchased from Calbiochem/Novabiochem (Bad Soden, Germany), Complete[®] was from Roche Diagnostics GmbH (Mannheim, Germany). Rabbit anti-Akt antibody, monoclonal mouse anti-phospho Akt (Ser473) antibody, rabbit anti-phospho Akt (Ser473) antibody, rabbit anti-phospho Bad (Ser136) antibody, rabbit anti-phospho Bad (Ser112) antibody and rabbit anti-Bad antibody were from New England Biolabs GmbH (Frankfurt, Germany). Goat anti-rabbit-IgG was from Dianova (Hamburg, Germany) and goat anti-mouse-IgG1 antibody conjugated to horseradish peroxidase was from BIOZOL (Eching, Germany). The secondary antibody (Alexa Fluor 488 goat anti-mouse) and rhodamine-conjugated phalloidin were from MoBiTec (Göttingen, Germany), [γ -³²P]-ATP was from Amersham-Pharmacia (Braunschweig, Germany) and the ApopTaq[®] peroxidase *in situ* apoptosis detection kit was from InterGen (New York, USA). All other materials were purchased from either Sigma (Taufkirchen, Germany) or VWR International[™] (Munich, Germany).

Animals and orthotopic liver transplantation

Syngeneic male Lewis rats (donors: 207±12 g; recipients: 276±18 g) were purchased from Charles River Wiga (Sulzfeld, Germany) and housed in a temperature- and humidity-controlled room under a constant 12 h light/dark cycle. Animals had free access to water and rat chow (SSniff, Soest, Germany), but were fasted with free access to water 12 h prior to the operation. All animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" published by the National Institute of Health (NIH publication 86-23 revised 1985). Studies were performed with the permission of the government authorities.

In transplantation experiments donor animals obtained either an intravenous infusion of NaCl or ANP (5 µg/kg). For investigation of signalling pathways additional rats were treated with wortmannin (16 µg/kg), DMSO (0.1%), or a combination of ANP and wortmannin for 20 min prior to hepatectomy. Blood pressure and heart rate were continuously monitored by a catheter in the carotid artery. A jugular venous catheter served to apply substances and substitute plasma volume. Body temperature was kept at 36.5 - 37.5 °C by means of a heating pad. Donor liv-

ers were preserved by retrograde aortal flush with 10 mL UW-solution and stored at 4 °C for 24 h. Before implantation, the livers were rinsed with cold Ringer's solution (10 mL) *via* the portal vein at a hydrostatic pressure of 10 cm H₂O. Orthotopic liver transplantation was performed as previously described^[4]. Grafted livers were simultaneously reperfused after completion of the arterial anastomosis. Portal clamping time was less than 20 min in all experiments.

The graft's common bile duct was cannulated with a PE-tube and bile was collected with its volume determined. Plasma samples (400 µL) were obtained from the recipient before hepatectomy and 60 and 120 min after reperfusion of the transplanted liver. The volume of the blood drawn was replaced by saline. After starting reperfusion, rats received 1.0 mL of albumin (5%) and 0.5-1.0 mL sodium bicarbonate to maintain blood pressure and physiological pH. To avoid fluid loss and drying of the liver, the abdominal cavity was covered with Saran wrap. After 20 min preconditioning, 24 h cold ischemia, or 120 min reperfusion, organs were placed in 4% paraformaldehyde or snap frozen in liquid nitrogen.

Histological analysis of tissue

Liver samples were fixed in paraformaldehyde, embedded in paraffin, and cut into 6µm sections. TUNEL positive cells^[15] were determined by staining of liver sections with the ApopTaq[®] peroxidase *in situ* apoptosis detection kit according to the manufacturer's instructions. The ApopTaq[®] staining results were evaluated in combination with morphological criteria. For counting of apoptotic cells, an area of 1.96 mm² (approximately 4000 hepatocytes) was observed by a Leitz Laborlux S microscope.

For analysis of pAkt snap frozen livers were cut into 6 µm sections. Slices were dried overnight at room temperature. Staining of pAkt was performed using the monoclonal anti-pAkt antibody in 0.2% BSA as the primary antibody (1 h, RT) and Alexa Fluor 488 goat anti-mouse antibody (1 h, RT). Sections were observed under confocal laser microscope (LSM 510 Meta, Zeiss, Jena, Germany). All histological evaluations were performed in a blinded fashion.

Homogenization of liver tissue for Western blotting

Fifty mg of liver tissue was homogenized in 1.5 mL of lysis buffer (50 mM Tris-HCl, 5 mM EGTA, 1 mM PMSF, 1 mM Na-vanadate pH 7.0, 40 µL Complete[®]) containing 1% Triton[®] X-100 (Roth, Karlsruhe, Germany) with a dounce homogenizer. After centrifugation of samples (14 000 r/min, 15 min, 4 °C), the supernatant was diluted with SDS-containing sample buffer. Samples were stored at -20 °C for Western blotting.

Immunoprecipitation

Liver tissue was homogenized as described above, 100 µg of protein in 100 µL lysis buffer was incubated with 2.5 µL of primary antibody (rabbit anti-Bad) shaking overnight at 4 °C. The antibody-antigen complex was precipitated by incubation with 10 µL of washed agarose-A-beads for 2 h, followed by centrifugation. The beads were washed three times with cold lysis buffer, and resuspended in 40 µL of 3

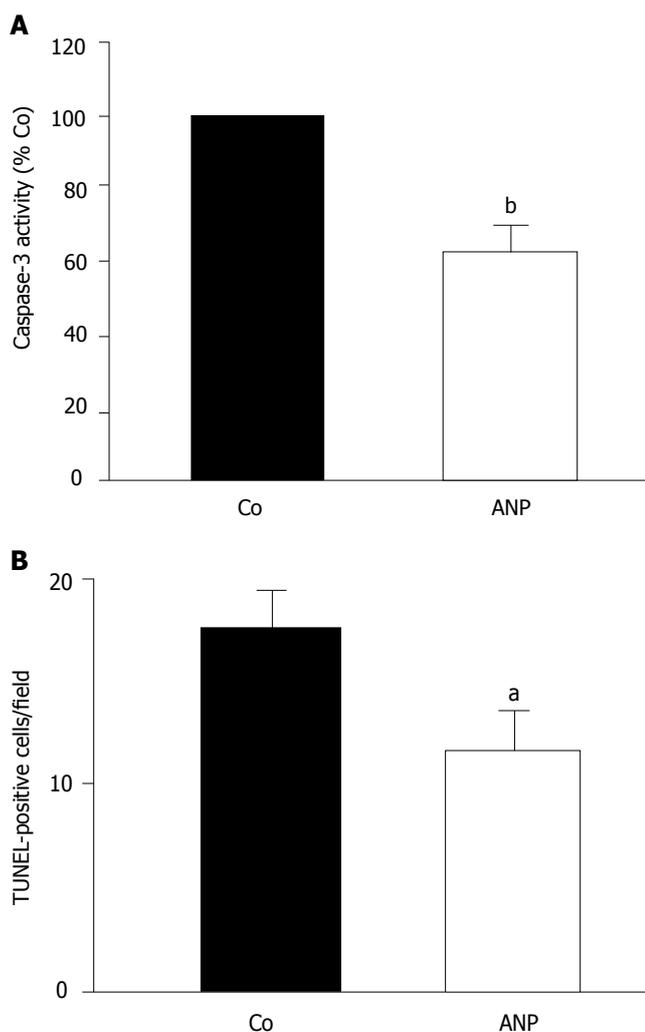


Figure 1 Effect of ANP pretreatment on apoptotic cell death after 24 h ischemia. Animals received an intravenous infusion of NaCl (Co) or ANP (5 µg/kg) for 20 min and livers were kept in cold UW-solution for 24 h. **A:** Caspase-3 activity: Snap-frozen tissue was homogenized and caspase-3 like activity was determined as described. Data were expressed as percentage of caspase-3 activity in untreated livers (100%). Columns show mean ± SE, *n*=4 animals in each group. ^b*P*<0.01 vs NaCl treatment. **B:** TUNEL assay: Snap-frozen tissue was cut into 6 µm slices. TUNEL staining was performed as described and apoptotic liver cells per field of view (1.96 mm²) were counted. Results were expressed as means ± SE, *n*=4 in each group. ^a*P*<0.05 vs NaCl treatment.

× SDS-containing sample buffer. After addition of 40 µL 1× sample buffer samples were boiled at 95 °C for 5 min followed by centrifugation.

Western blotting

Homogenized livers were treated as described before. Proteins in total liver homogenates or immunoprecipitates were separated by SDS-Page and visualized after electrophoretic transfer via binding of specific primary and HRP-conjugated secondary antibodies followed by chemoluminescent detection. Detection and quantification were performed with a Kodak image station (NEN, Cologne, Germany).

PKA activity assay

A commercial PKA assay kit was used. Samples (100 µg) were homogenized in “extraction buffer” and centrifuged

as described in the manufacturer’s manual. Supernatants were used for measurement of phosphorylation activity of PKA by *in vitro* phosphorylation of the specific peptide substrate kemptide with [³²P]-ATP.

Caspase-3 like activity assay

After homogenization of 100 mg liver tissue in 1 mL lysis buffer (25 mM HEPES, 5 mM MgCl₂, 1 mM EGTA, pH 7.5, Complete[®]), samples were centrifuged (14 000 r/min, 10 min, 4 °C). Caspase-3 like activity in the supernatants was determined as previously reported^[3]. Generation of free fluorescent 7-amino-4-trifluoro-methylcoumarin was measured with a Fluostar analyser (BMG GmbH, Offenburg, Germany).

Statistical analysis

All experiments were performed at least three times per treatment group. Results were expressed as mean ± SE. Statistical significance between groups was determined with one sample or Student’s *t* test using GraphPad Prism[®] Version 3.02 for Windows (GraphPad Software Inc., San Diego, USA). *P*<0.05 was considered statistically significant.

RESULTS

Reduction of apoptotic cell death *in vivo* induced by ANP-pretreatment

Apoptotic processes were monitored by measuring caspase-3 activity and TUNEL staining of liver sections. After 24 h cold ischemia, an increase of hepatic caspase-3 activity (40.5%) compared to sham operated animals was observed, which sustained after 2 h reperfusion (25%). Figure 1A demonstrates a significant attenuation of caspase-3 activity by ANP-pretreatment, which was also observed in 2 h reperused organs after 24 h cold storage.

TUNEL-staining of corresponding liver sections confirmed these data. After 24 h cold ischemia apoptotic cells were most prominent. Both hepatocytes and endothelial cells showed positive TUNEL staining combined with characteristic apoptotic morphology.

Figure 1B shows that preconditioning of donor livers with ANP (5 µg/kg) significantly decreased the number of TUNEL-positive cells after 24 h cold ischemia. A similar although less pronounced effect was observed after 2 h reperfusion.

Activation of PI-3-Kinase/Akt pathway induced by ANP pretreatment

Infusion of ANP (5 µg/kg) for 20 min into the donor animals resulted in a marked increase of phosphorylated Akt (pAkt) in the liver, which was abrogated in the presence of the PI-3-Kinase inhibitor wortmannin (Figure 2A).

Antiapoptotic effects of ANP induced by Akt activation

Figure 2B demonstrates that application of wortmannin (16 µg/kg) prior to ANP infusion completely blocked the inhibition of caspase-3 activity by ANP seen in ischemic liver tissue (24 h). Thus, the PI-3-Kinase pathway was intimately involved in the antiapoptotic effect of ANP-

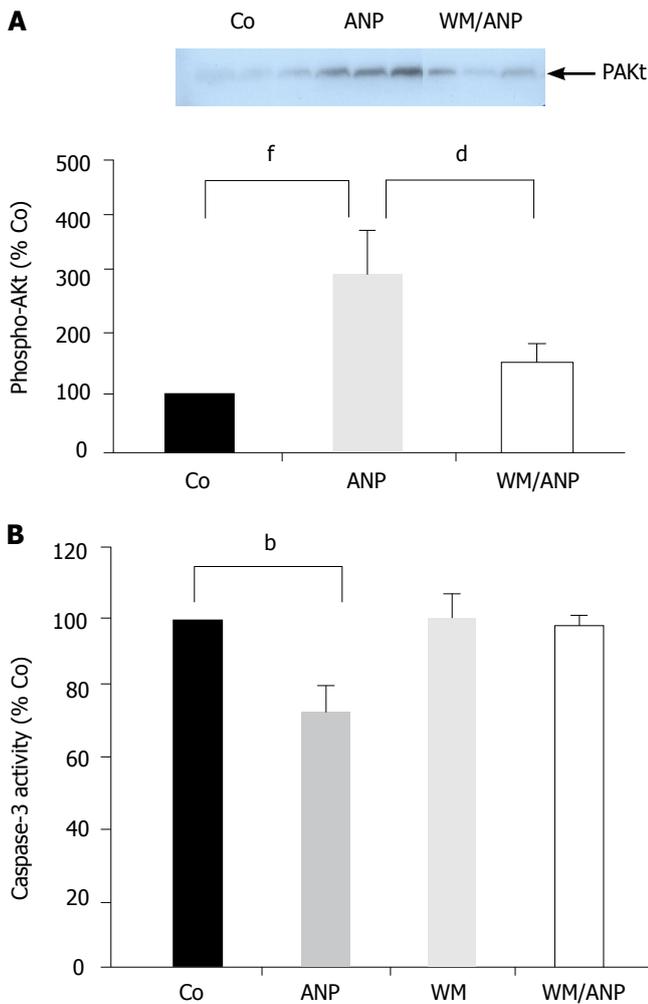


Figure 2 Effect of ANP-preconditioning on PI-3-kinase/Akt pathway. **A:** After intravenous infusion of NaCl (Co), ANP (5 µg/kg), wortmannin (WM, 16 µg/kg), or a combination of both (WM/ANP) for 20 min livers were excised and snap frozen. pAkt was examined via Western blotting. Data obtained from densitometry were presented as percent of Co (100%) and expressed as mean ± SE, $n=4$ in each group. $^{\ast}P < 0.01$ vs ANP treatment; $^{\ast\ast}P < 0.001$ vs Co treatment. **B:** Animals were treated as described under A. After 24 h ischemia livers were homogenized and caspase-3 activity was determined as described before. Data were expressed as percent of caspase-3 activity in untreated livers (Co, 100%) and mean ± SE, $n=4$ in each group. $^{\ast}P < 0.01$ vs WM treatment.

preconditioning.

Translocation of activated Akt to plasma membrane induced by ANP

Analysis of pAkt in liver tissue by confocal microscopy confirmed that ANP-pretreatment could lead to a marked increase of pAkt. Interestingly, confocal microscopy revealed a strong localization of pAkt at the plasma membrane of hepatocytes (Figures 3A, 3B). Cotreatment of animals with wortmannin abrogated both the increase as well as the plasma membrane localization of pAkt induced by ANP (Figure 3C).

Phosphorylation of antiapoptotic Bad due to Akt activation by ANP

To further examine the antiapoptotic signalling in the liver induced by ANP *in vivo*, a pivotal downstream target of pAkt (the Bad protein) was investigated. A representative Western blot indicated that Bad was phosphorylated at

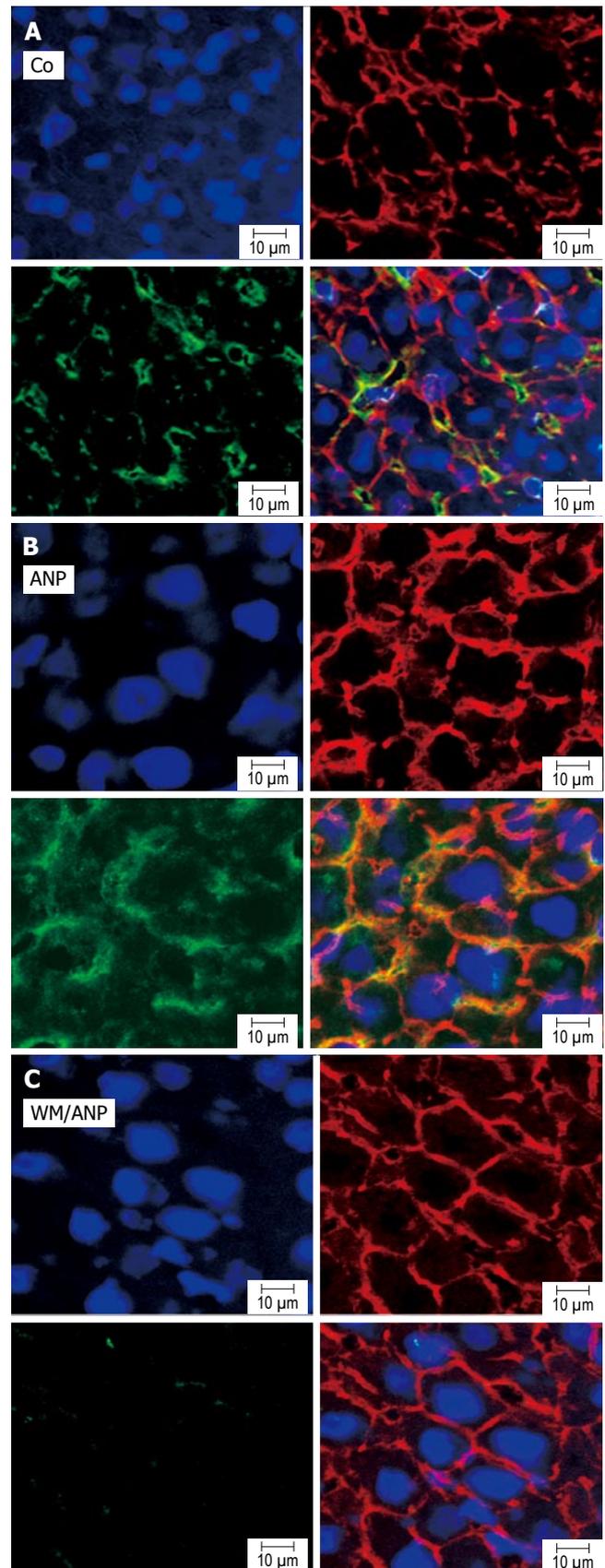


Figure 3 Effect of ANP-preconditioning on distribution and amount of pAkt in liver by confocal microscopy. Animals were infused with NaCl (Co, **A**), ANP (**B**), or a combination of ANP and wortmannin (WM/ANP, **C**) for 20 min. Livers were snap-frozen, cut into 6 µm slices and stained with rhodamine-conjugated phalloidin (F-actin, cytoskeleton, red), HOECHST dye (nuclei, blue), and anti-pAkt antibody, followed by incubation with Alexa Fluor 488 goat anti-mouse antibody (green) as described before. Liver sections were observed under confocal laser microscope ($\times 63$ amplification).

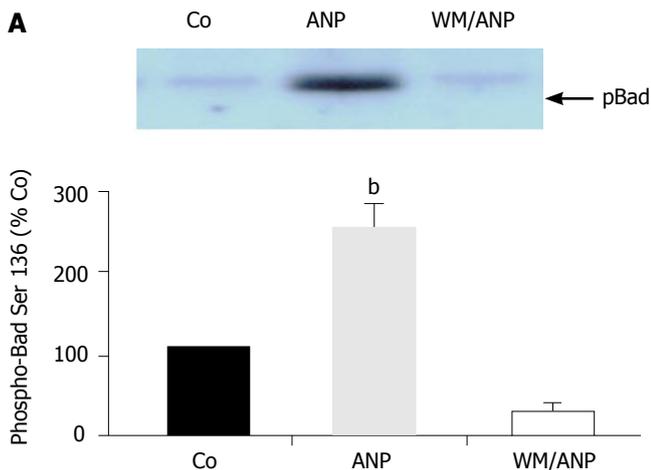


Figure 4 Effect of ANP-preconditioning on phosphorylation of Bad at Ser136. After intravenous infusion of NaCl (Co), ANP (5 μ g/kg), or a combination of ANP and wortmannin (WM/ANP) for 20 min livers were excised and snap frozen. Homogenized livers were immunoprecipitated using anti-Bad antibody. Examination of pBad (Ser136) was done via Western blotting. Data obtained by densitometry were presented as mean \pm SE, $n=4$ in each group. Co was set as 100%. ^b $P < 0.001$ vs control.

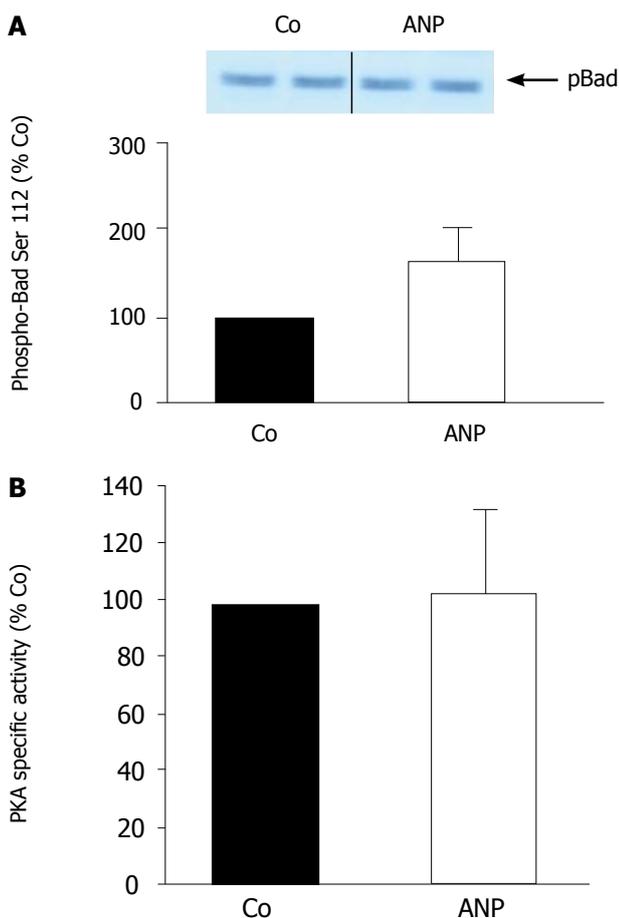


Figure 5 Effect of ANP pretreatment on phosphorylation of Bad at Ser112 and PKA-activity. **A:** Animals received an intravenous infusion of NaCl (Co) or ANP (5 μ g/kg) for 20 min. Homogenized livers were immunoprecipitated using anti-Bad antibody. Examination of phospho-Bad (Ser112) was done via Western blotting. Data obtained in densitometric analysis were presented as mean \pm SE, $n=3$ in each group. Co was set as 100%. **B:** PKA activity was determined in liver homogenates used under (A) by *in vitro* phosphorylation as described. Results were expressed as percent of PKA activity in untreated livers and mean \pm SE, $n=4$ in each group.

Ser136 by pAkt induced by ANP (Figure 4). ANP *in vivo* did not activate PKA and consequently phosphorylated Bad at Ser112 (Figure 5A, Figure 5B).

DISCUSSION

This study showed that pretreatment of rats with ANP could reduce apoptotic processes mainly occurring after 24 h cold ischemia in orthotopic rat liver transplantation. In this setting ANP activated the PI-3-Kinase/Akt pathway leading to Bad phosphorylation and inhibition of caspase-3 activity, revealing that the PI-3-Kinase/Akt pathway is an important cytoprotective mechanism in IRI *in vivo*.

PI-3 Kinases are heterodimers composed of a catalytic subunit (p110) and a regulatory subunit (p85) activated by receptors with protein tyrosine kinase activity as well as G-protein-coupled receptors^[16, 17]. Various growth factors such as IGF-1 activate PI-3-Kinase leading to the generation of phosphoinositides [PI(3,4,5)P₃] and activation of Akt, a 57 kDa Ser/Thr kinase. PI-3-kinase regulates Akt kinase activity by generated phospholipids^[16, 17]. One mechanism is through the direct binding of phosphoinositides to the pH domain of Akt, which seems to be critical for the *in vivo* activation of Akt. A consequence of Akt binding to phospholipids is the translocation of Akt from cytoplasm to the inner surface of plasma membrane^[16, 17]. This translocation which we also observed in hepatocytes pretreated with ANP, is important for Akt activation. In fact, protection from apoptotic stimuli in cardiomyocytes and liver has been demonstrated using adenovirally mediated gene transfer of membrane-targeted constitutively activated Akt^[18-21]. However, the constitutively activated form of Akt targeted to the cell membrane results in supraphysiological levels of kinase activity having the potential to induce oncogenic transformation. These issues of feasibility and safety concerning an Akt gene therapy are certainly much less relevant in a setting of Akt activation by endogenous stimulators such as ANP or others^[22-25].

Up to now, protective effects of ANP against IRI have only been demonstrated in *ex vivo* and *in vitro* settings such as isolated perfused rat liver^[3], isolated liver cells^[13, 26], and isolated ischemic heart^[27]. The underlying mechanisms seem to be highly dependent on the experimental model used. For instance, as recently described in the *ex vivo* model, ANP protects hepatocytes from apoptosis after cold ischemia by activation of PKA^[28]. ANP treatment of hypoxic isolated rat hepatocytes confers its cytoprotection *via* PKC σ and activation of p38 MAPK^[13]. This difference might be explained by different modes of cell death analyzed. Focusing on apoptotic cell death, our data showed that the *in vivo* setting revealed a different protective signaling, i.e. activation of the PI-3-kinase/Akt pathway compared to the isolated perfused rat liver subjected to IR. Accordingly, activation of Akt can also protect livers against warm IRI^[21]. Importantly, both pathways, PKA activation and Akt activation, lead to phosphorylation and inactivation of Bad protein, suggesting that this protein plays a key role in ANP-induced prevention of ischemia-induced apoptosis.

Bad is a member of the family of Bcl-2 proteins func-

tioning as apoptosis-regulating factors^[29, 30]. Bad in its unphosphorylated form binds to and inactivates antiapoptotic proteins such as Bcl-2 and Bcl-XL leading to proapoptotic functions. Phosphorylation of Bad at either of the two potential sites (Ser112, Ser136) causes Bad to dissociate from Bcl-2 or Bcl-XL respectively and to associate instead with cytoplasmic 14-3-3 proteins preventing Bad from dephosphorylation^[16]. Akt phosphorylates Bad at Ser136 *in vivo* and mutation of Bad Ser136 to alanine abrogates the blocking effect of Akt in Bad-induced apoptosis. In any case, Bad plays a key role in the Akt survival signalling since Bad phosphorylation leads to prevention of cytochrome C release from mitochondria, a hallmark of apoptosis induction^[16].

We would like to point out that besides acting through Bad phosphorylation Akt brings about its function also by a variety of other downstream targets, such as caspase-9, forkhead family members or the eNOS enzyme^[16]. Akt-mediated phosphorylation and activation of the eNOS enzyme have been reported to be involved in the antiapoptotic effect of insulin in myocardial IR^[25]. In line with this report a blockade of the NO synthase worsens hepatic apoptosis and liver transplant preservation injury^[31]. Since we did not observe increased phosphorylation of eNOS in ANP-pretreated livers (data not shown), this mechanism does not contribute to the antiapoptotic action of ANP.

In conclusion, Bad inactivation by phosphorylation should be considered as an important therapeutic target preventing IRI during liver transplantation.

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BASIC RESEARCH

Relationships between mucinous gastric carcinoma, MUC2 expression and survival

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with decreased defensin expression in ulcerative colitis in addition to high expression of cytokines. The low incidence of pouchitis in FAP pouches correlates with increased expression of hBD-1 β - defensin in association with low cytokine levels.

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Key words: Innate immunity; Ulcerative colitis; Defensins; Pouchitis; Cytokines

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<http://www.wjgnet.com/1007-9327/12/1056.asp>

Abstract

AIM: Pouchitis develops in ileoanal pouches in up to 50% of patients with ulcerative colitis during the first 10 years after pouch surgery while being rare in patients after proctocolectomy for familial adenomatous polyposis coli (FAP) syndrome. Defensins are major components of the innate immune system and play a significant role in gastrointestinal microbial homeostasis. Pouch defensin and cytokine expression were correlated with states of pouch inflammation to study their role in pouchitis.

METHODS: Patients with ulcerative colitis and FAP syndrome were stratified into groups with pouches after surgery, pouches without or with pouchitis. Biopsies from terminal ileum from a healthy intestine or from normal terminal ileum of patients with ulcerative colitis served as controls. mRNA from pouches and controls was analysed for defensin and cytokine expression.

RESULTS: Expression of defensins was increased in all pouches immediately after surgery, compared to ileum of controls. Initially, pouches in ulcerative colitis revealed higher defensin expression than FAP pouches. Defensin expression declined in both patient groups and increased again slightly in pouchitis in patients with ulcerative colitis. FAP pouches without pouchitis had strong expression of β -defensin hBD-1, while all other defensins remained at low levels. Cytokine expression in ulcerative colitis pouches was high, while FAP pouches showed moderately elevated cytokines only after surgery.

CONCLUSION: Development of pouchitis correlates

INTRODUCTION

Pouchitis is a major long term complication after proctocolectomy and ileoanal pouch anastomosis. Up to 50% of patients with ulcerative colitis experience at least one episode during the first ten years after surgical pouch construction while pouches in patients with familial adenomatous polypous (FAP) syndrome have pouchitis rates below 5%^[1,2]. The causes of primary pouchitis remain uncertain, while secondary pouchitis is usually being caused by conditions that respond to surgical treatment (e.g. fistulas, local ischemia). Pouchitis develops when the faecal stream is re-diverted towards the pouch after closure of a protective ileostomy indicating the involvement of bacteria in the pathomechanism of pouchitis^[3] which is further substantiated by the effective treatment of pouchitis with antibiotics^[4].

Ulcerative colitis is a chronic inflammatory disorder of the intestine of unknown aetiology. Immunologic and genetic determinants appear to be major disease factors, but environmental factors and impaired host defense mechanisms emerge to be of significant importance^[5,6,7]. The epithelial defense lines against microbial insults comprise co-ordinated actions of the innate and the acquired immune responses. Thus, defects of these mechanisms might result in an overgrowth of normal colonic flora with increases in aerobic bacteria in addition to the appearance of atypical bacteria and fungi^[8]. Finally, local invasion of microbials and induction

Table 1 Patients and sample characteristics

| | UC pouch before closure of ileostomy | UC pouch in use, no pouchitis | UC pouchitis | FAP pouch before closure of ileostomy | FAP pouch in use, no pouchitis | FAP pouchitis | Healthy control | UC control term. ileum without inflammation |
|-----------------------|--------------------------------------|-------------------------------|--------------|---------------------------------------|--------------------------------|---------------|-----------------|---|
| Patients (n) | 10 | 15 | 11 | 6 | 14 | 7 | 23 | 35 |
| Age(yr) | 17-36 | 19-45 | 27-50 | 17-27 | 19-45 | 22-36 | 17-53 | 18-59 |
| Sex(f/m) | 5/5 | 8/7 | 4/7 | 4/2 | 10/4 | 4/3 | 12/11 | 23/12 |
| Pouch Classification: | | | | | | | | |
| Moskowitz | 2-4 | < 4 | > 4 | 2-4 | < 4 | > 4 | - | - |

PDAI: pouchitis disease activity index (13); Values in points: <7: no pouchitis; >7: pouchitis

Moskowitz: histologic classification of inflammatory changes in pouches (14). Values in points: <4 no pouchitis; >4: pouchitis

UC: ulcerative colitis

of inflammatory responses causes self-destructive injury.

The FAP syndrome is caused by genetic changes leading to growth of colonic polyps. For both disorders, ulcerative colitis and FAP syndrome, ileoanal pouch procedure is a valuable therapeutic option to prevent development of colorectal cancer.

α - and β -defensins are major components of the epithelial mammalian innate immune system^[9]. Defensins are small cationic peptides with high activity against a variety of microbes, encoded by genes and some are regulated in response to challenge with bacterial antigens. Gastrointestinal α -defensins (HD5 and HD6) are almost exclusively expressed in and secreted from Paneth cells of the small intestine, while β -defensins (hBD-1, hBD-2, hBD-3) are secreted by virtually all gastrointestinal epithelial cells to a varying extent^[10,11]. Defensins are envisaged to play significant roles in intestinal microbial homeostasis and in the primary defence against enteric and systemic infections^[9,12]. In the present study we analysed defensin expression in ileoanal pouches of patients with ulcerative colitis and FAP syndrome. Pouches were stratified into groups with ileostoma-protected pouches after surgery, and pouches (after closure of the ileostomy) without or with pouchitis. Expression data of antimicrobial peptides were related with expression of cytokines and the pouch groups to analyse the potential role of defensins as protectors for the development of pouchitis.

MATERIALS AND METHODS

Patients

Pouch patients with ulcerative colitis or FAP syndrome as the underlying disease were included. After construction of ileoanal pouches the patients were followed by pouchoscopy and biopsies were collected for histology and RNA analysis. Pouches from patients with ulcerative colitis or FAP syndrome were further stratified into the following groups (Table 1): 1) pouches soon after surgery (without faecal load and with protective ileostoma), 2) pouches after closure of the ileostoma, no signs of pouchitis, 3) pouches with pouchitis. Severity of pouchitis was scored by the pouchitis disease activity index (PDAI), comprising clinical, endoscopic and histologic features^[13]

and the Moskowitz score to classify histology of pouch biopsies^[14].

Biopsies from control patients or from control patients with ulcerative colitis were collected from a macroscopically and histologically healthy terminal ileum. Patients characteristics were comparable between controls, ulcerative colitis and FAP groups, as shown in Table 1.

Two biopsies were examined by histology and two others were immediately snap-frozen in liquid nitrogen for RNA analysis. Microscopy (not shown) of pouch biopsies showed features of metaplasia in all pouches when pouches had been exposed to faeces for several months. There was an increase of mucin containing cells and with a reduction of villi in the ileal mucosa as previously reported^[15,16,17]. Electron microscopy also demonstrated an increase of mucin containing cells while Paneth cells remained stable in number and morphology (not shown). Changes were most pronounced in biopsies from pouches with the most severe signs of pouchitis in endoscopy.

The study was approved by the Ethics Committees of the University of Kiel, Germany and the University of Poznan, Poland (Ref.No. A158/01). All patients gave their written informed consent prior to investigation.

RNA isolation and cDNA synthesis

Total RNA was extracted from frozen biopsies using silica gel-based spin columns (RNeasy Kit, QIAGEN, Hilden, Germany). Genomic DNA was digested by thorough treatment with deoxyribonuclease I (QIAGEN, Hilden, Germany). Reverse transcription of 2 μ g RNA was performed using 0,5 μ g oligo(dT)₁₅-primers (You prime First strand cDNA synthesis kit; Amersham Biosciences, Freiburg, Germany) following the manufacturers instructions.

Primer

Specific intron spanning primers were designed based on the published mRNA sequences using the Primer3 software^[18] (also available under <http://www-genome.wi.mit.edu/genome-software/other/primer3.html>). Oligonucleotides were obtained from Sigma ARK (Darmstadt, Germany), sequences and product sizes for each primer pair used are depicted in Table 2.

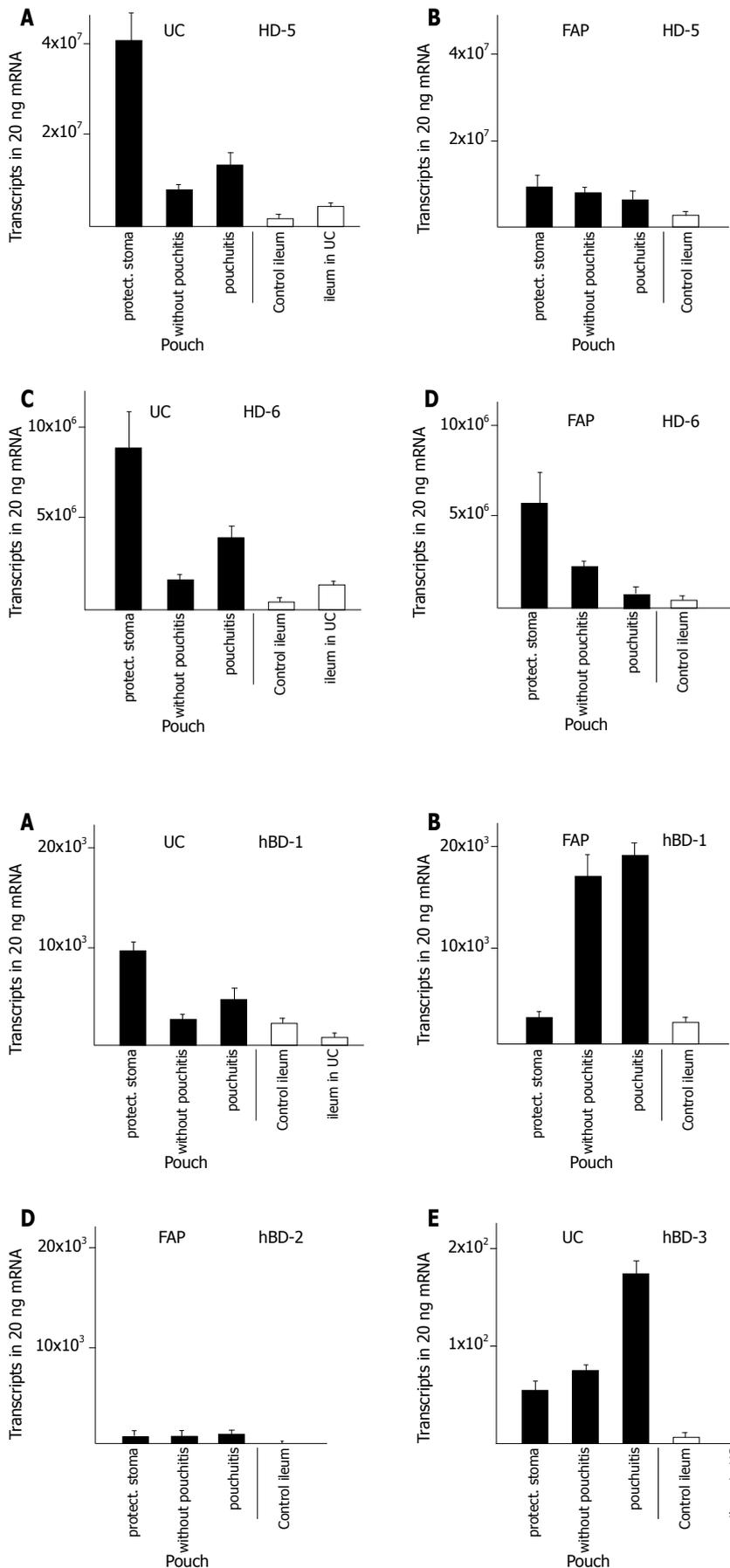


Figure 1 α -defensin mRNA expression in pouches of patients with ulcerative colitis (UC) and FAP syndrome. mRNA was extracted from biopsies, reverse transcribed and analysed by quantitative real time RT-PCR. Expression of HD5 (A, B) and HD6 (C, D) in patients with UC (A, C) and FAP (B, D). Control ileum was from healthy individuals. Ileum in UC was from patients with active UC but with completely normal ileum. Values represent mean \pm SE.

Figure 2 β -defensin mRNA expression in pouches of patients with ulcerative colitis (UC) and FAP syndrome. mRNA was extracted from biopsies, reverse transcribed and analysed by quantitative real time RT-PCR. Expression of hBD-1 (A, B) and hBD-2 (C, D) and hBD-3 (E, F) in patients with UC (A, C, E) and FAP (B, D, F). Control ileum was from healthy individuals. Ileum in UC was from patients with active UC but with completely normal ileum. Values represent mean \pm SE.

Table 2 Nucleotide sequences of PCR primer (source: human)

| Primer | Orientation | Sequence | PCR product size(bp) | GeneBank accession |
|--------------|-------------|--|----------------------|--------------------|
| hBD-1 | Sense | 5'-TTG TCT GAG ATG GCC TCA GGT GGT AAC | 253 | NM005218 |
| | Antisense | 5'-ATA CTT CAA AAG CAA TTT TCC TTT AT | | |
| hBD-2 | Sense | 5'-ATC AGC CAT GAG GGT CTT GT | 173 | AF448141 |
| | Antisense | 5'-GAG ACC ACA GGT GCC AAT TT | | |
| hBD-3 | Sense | 5'-AGC CTA GCA GCT ATG AGG ATC | 206 | NM018661 |
| | Antisense | 5'-CTT CGG CAG CAT TTT CGG CCA | | |
| HD5 | Sense | 5'-GCC ATC CCT GCT GCC ATT C | 241 | NM 021010 |
| | Antisense | 5'-AGA TTT CAC ACC CCG GAG A | | |
| HD6 | Sense | 5'-CCT CAC CAT CCT CAC TGC TGT TC | 266 | NM001926 |
| | Antisense | 5'-CCA TGA CAG TGC AGG TCC CAT A | | |
| TNF α | Sense | 5'-TCA GCT TGA GGG TTT GCT ACA A | 100 | NM000594 |
| | Antisense | 5'-TCT GGC CCA GGC AGT CAG ATC | | |
| IL-1 β | Sense | 5'-CCA GCT ACG AAT CTC CGA CCA CCA CTA C | 600 | NM000576 |
| | Antisense | 5'-TGC TTG AGA GGT GCT GAT GTA CCA GTT G | | |
| IL-4 | Sense | 5'-AAC ACA ACT GAG AAG GAA ACC TTC T | 276 | NM000589 |
| | Antisense | 5'-GCT CGA ACA CTT TGA ATA TTT CTC | | |
| IL-6 | Sense | 5'-AGG AGC CCA GCT ATG AAC TCC TTC | 120 | NM000600 |
| | Antisense | 5'-TGG AAT CTT CTC CTG GGG GTA CTG | | |
| IL-8 | Sense | 5'-ATG ACT TCC AAG CTG GCC GTG GC | 307 | NM000584 |
| | Antisense | 5'-TCT CAG CCC TCT TCA AAA ACT TC | | |

Quantification of gene expression by real time PCR

Real-time RT-PCR analyses were performed in a fluorescence temperature cycler (LightCycler, Roche Molecular Biochemicals) according to the manufacturer's instructions. Standard curves for each specific defensin were constructed by cloning the purified PCR-products containing the target sequence into pCR-Blunt II-TOPO vector with 3519 bp (Invitrogen). Clones were sequenced on ALP Express (Pharmacia, Uppsala, Sweden). Concentration of the reference plasmid was measured spectrophotometrically and converted into number of copies / μL by using the formula: number of copies/ μL = $(X \text{ g}/\mu\text{L} \text{ plasmid DNA} / Y \times 660) \times 6.023 \times 10^{23}$, where X is the measured plasmid concentration, Y is the number of nucleotide pairs for the plasmid plus the size of the insert, 660 g/mol is the average molecular weight of a nucleotide pair, and 6.023×10^{23} is Avogadro's number (number of copies/mol). To generate standard curves, the plasmids were serially diluted (1: 10) over the appropriate concentration range. The solvent included unrelated, defensin- sequence free cDNA in a final concentration of 10 ng/ μL . To achieve a reliable standard curve for each measured defensin, the plasmids were PCR- amplified in three replicates for each standard dilution point over the complete range (10^7 to 10^0). Standard curves were measured repeatedly to calculate averages. The reverse transcribed cDNA from the biopsies corresponding to 20 ng of RNA served as template in a 10 μL PCR reaction containing 4 mmol/L MgCl_2 , 0,5 $\mu\text{mol/L}$ of each primer and 1x LightCycler-Fast Start DNA Master SYBR Green I mix (Roche Molecular Biochemicals, Mannheim, Germany). Samples were loaded into capillary tubes and incubated in the fluorescence thermocycler (LightCycler) with initial denaturing at 95 °C for 10 min, followed by 45 cycles, each cycle consisting of 95 °C for 15 s for denaturation, primer

specific annealing temperature for 10 s, and 72 °C for 10s for elongation. SYBR Green I fluorescence was detected at the end of each cycle to monitor the amount of PCR product formed during the cycle. At the end of each run, melting curve profiles were produced (cooling the samples to 65 °C for 15 s and then heating slowly at 0.2 °C/s to 95 °C with continuous measurement of fluorescence) to exclude non-specific amplification of transcripts. The absolute mRNA transcript number in each sample was calculated by use of calibration curves. Identical results were obtained in control experiments when the standard plasmids were mixed with cDNA obtained from a variety of sources including cDNA from cultured intestinal mouse STC-1 cells which do not contain defensin transcripts (not shown).

RT-PCR of cytokines

Reverse transcribed cDNA from biopsies corresponding to 20 ng RNA was used as template in a 10 μL PCR reaction using Taq polymerase in a Thermal cycler (Gene Amp PCR System 2400, Perkin Elmer). PCR started with initial denaturation at 95 °C for 10 min, followed by 30 cycles each consisting of denaturation at 95 °C for 45 s, annealing at 60 °C for 45 s and elongation at 72 °C for 60 s. Elongation in the final PCR cycle was for 7 min. PCR products were resolved by 8% agarose gel electrophoresis

RESULTS

We established control groups of normal ileum from healthy patients and ileum from patients with active ulcerative colitis but without macroscopic or microscopic involvement in order to obtain parameters to which defensin expression in diseases could be correlated. The expression of α -defensins HD5 (Figure 1A) and HD6 (Figure 1C) in ileum of patients with ulcerative colitis was

Table 3 Cytokine expression in ulcerative colitis or FAP pouches

| Cytokine | Controls | | | Pouches | | | | |
|--------------|---------------|-------------|--------------|----------------------|--------------|---------------|-----------------------|---------------|
| | Ileum control | Ileum in UC | UC ileostoma | UC without pouchitis | UC pouchitis | FAP ileostoma | FAP without pouchitis | FAP pouchitis |
| TNF α | + | (+) | +++ | ++ | ++ | + | + | + |
| IL-1 β | - | ++ | +++ | +++ | +++ | +++ | ++ | + |
| IL-4 | - | - | (+) | (+) | (+) | + | (+) | (+) |
| IL-6 | - | - | + | + | (+) | (+) | (+) | (+) |
| IL-8 | (+) | ++ | ++ | ++ | ++ | ++ | + | (+) |

UC: ulcerative colitis; FAP familial adenomatous polyposis cancer syndrom. Samples were analysed by rt-PCR.

+++: strong expression in all samples

++: medium expression in all samples

+: weak expression in most samples

(+): weak expression in some samples

increased by two to three fold, resp, compared to ileum of healthy controls. The β -defensins HBD-2 (Figure 2C) or hBD-3 (Figure 2E) were present at very low levels in the healthy ileum. HBD-2 expression was increased (from 3 ± 1 to 149 ± 110 transcripts per 20 ng mRNA) in the ileum in patients with ulcerative colitis but remained still at insignificant levels, and hBD-3 remained unchanged. Surprisingly, hBD1 (Figure 2A) expression in the not affected ileum of patients with ulcerative colitis is reduced to levels below 30% of hBD-1 expression in a normal ileum.

Expression of both α - and β - defensins in pouches of ulcerative colitis and FAP patients was most pronounced during the first three to nine months after surgical construction. Pouches at that time were not exposed to the faecal stream because of the presence of an upstream protective ileostoma. α -Defensin expression was increased by 5- fold (HD6), and 10- fold (HD5), compared with the expression in the not affected ileum in patients with ulcerative colitis (Figure 1). In pouches of ulcerative colitis patients, hBD-1 showed a 15- fold increase compared to the ileum in patients with ulcerative colitis (Figure 2A). HBD-2 is virtually absent in the normal ileum, but in pouches after surgery hBD-2 is expressed at 18×10^3 copies per 20 ng mRNA which is a strong increase compared to normal ileum mucosa or to the ileum of patients with ulcerative colitis (Figure 2C). HBD-3 is expressed at very low levels in the ileum or in healthy or inflamed pouches while a slight increased expression is found in pouches after surgery (Figure 2F).

When pouches in ulcerative colitis patients are in use after closure of the protective ileostoma and pouchitis was not present, HD5 (Figure 1A), HD6 (Figure 1C), hBD-1 (Figure 2A), and hBD-2 (Figure 2C) expression levels declined from the initially present high levels to levels similar to or slightly increased above (2- to 4- fold) the defensin expression level in ileum of patients with ulcerative colitis. When pouchitis develops, a rise in defensin expression is observed between 2- fold (HD5, Figure 1A; HD6, Figure 1C; hBD-1, Figure 2A; hBD-3, Figure 2E) or 3- fold (hBD-2, Figure 2C) compared with the expression levels present in pouches without pouchitis.

Pouches of FAP patients after pouch surgery showed only minor changes of defensin expression, compared to normal ileum. Levels of HD5 and HD6 expression was increased 3- and 5 fold, respectively. (Figure 1B and D), while hBD-1 expression was unchanged (Figure 2B). HBD-2 and hBD-3 expression was increased, but total expression levels remained at very low transcript numbers (Figure 2D, F). When pouches were exposed to the faecal stream, the initial increased HD5 (Figure 1B) and HD6 (Figure 1D) defensin expression levels declined, and hBD-2 expression remained stable (Figure 2D). Pouchitis in FAP pouches did not cause an increase of HD5 (Figure 1B) and HD6 (Figure 1D) expression as pouchitis in ulcerative colitis patients, and hBD-2 (Figure 2D) expression levels remained unchanged. In contrast to all other defensins, hBD-1 (Figure 2B) expression levels in FAP pouches after surgery remained identical to hBD-1 expression levels in the normal ileum. Furthermore, hBD-1 expression increased markedly (6- fold) in the group of FAP pouches without pouchitis compared with normal ileum and hBD1 expression slightly increased further in FAP pouches under pouchitis.

Cytokine expression in pouch biopsies showed significant differences between the various pouch groups. Pouches from ulcerative colitis patients after surgery had strong expression of the proinflammatory cytokines TNF- α , IL-1 β and the immunomodulatory cytokine IL-10, when compared with unaffected ileum from ulcerative colitis patients (Table 3). Expression of IL-8 was intermediate while IL-4 and IL-6 were poorly detectable. The expression of TNF- α , IL-1 β , IL-8 and IL-10 remained largely unchanged in pouches from patients with ulcerative colitis, irrespective of the presence or absence of pouchitis and indicating a predominant TH1 response in the pouch patients with ulcerative colitis. Pouches from FAP patients immediately after surgery showed a similar pattern of cytokine expression as measured in pouches in ulcerative colitis, although cytokine expression levels were lower in the FAP patients. Cytokine expression declined in FAP pouches exposed to faeces. Interestingly, cytokine expression was undetectable for IL-4, IL-6, IL-8, and IL-10 while very minor expression was found for TNF- α

and IL-1 β in FAP pouches with pouchitis.

DISCUSSION

Surgical construction of an ileoanal pouch results in massive increases of α - and β -defensin levels in the ileum type pouch mucosa. In general, the expression of defensins in pouches is significantly higher than in the normal ileum. Furthermore, pouches in ulcerative colitis initially show much higher α - and β -defensin levels than pouches in FAP. When pouches are in use and exposed to faeces, they undergo metaplastic changes^[15,16,17] and show reduced defensin expression compared to pouches early after surgery which are protected by an upstream ileostomy. In the presence of pouchitis, there is an up regulation of α - and β -defensins in the inflamed pouches of ulcerative colitis patients. In contrast, FAP pouches show a down regulation of the initially elevated α - defensin expression in pouches without or with pouchitis. The most prominent changes are observed in the expression of the β -defensin hBD-1, which is markedly upregulated in FAP pouches after closure of the protective ileostoma and expressed at low levels in pouches of patients with ulcerative colitis. Furthermore, cytokines in pouches of patients with ulcerative colitis are strongly expressed in all stages while FAP pouches show a transient and weaker cytokine expression.

The pathomechanisms of pouchitis remain undetermined with potential genetic, microbial, and immunologic causes^[2,5,6,7]. The most obvious difference between ileoanal pouch mucosa and the normal ileum is metaplasia of the typical ileum mucosa to a more or less colonic epithelial type^[15,16,17]. Pouch mucosa has immunohistochemical features and enzymatic markers of both ileum and colon, but complete metaplasia does not occur. Paneth cells remain stable in cell number over several months after pouch construction^[17]. The observed changes in defensin expression in the present study can only partially be correlated to histologic changes or colonic metaplasia. The healthy ileum has a much higher expression of HD5 and HD6 than a healthy colon and an identical expression of the β -defensins (unpublished data). The initial extreme upregulation of α - defensins and to a lesser degree of β defensins after pouch construction could be due to Paneth and epithelial cell stimulation but can not be correlated to metaplasia which occurs at later times. Furthermore, the decrease in α -defensin expression upon time in older pouches or pouchitis cannot be correlated with a decrease of Paneth cell number. Thus, α -defensin transcription appears to be regulated after pouch surgery by unknown mechanisms, which cause multiplication of defensin expression during this time.

Pouches in ulcerative colitis develop pouchitis at a much higher incidence than pouches in FAP^[1] which has been related to the elevated cytokine levels in ulcerative colitis^[19,20]. Except for TNF- α and IL-10, cytokine expression pattern is largely similar in the colon and in pouches of patients with ulcerative colitis^[21]. The expression of cytokines (TNF- α , IL-1 β , IL-8 and IL-10) in pouches of ulcerative colitis patients is steadily upregulated independent of the presence or absence of pouchitis, which further

contrasts to FAP pouches, where cytokines are expressed only during the initial time after pouch construction but not in older pouches or in pouchitis. Regulation of defensin expression by TNF α , IL-1 α and IL-1 β cytokines has been described for hBD-2, hBD-3, and HD5 in cell culture systems, while expressional changes of hBD-1, hBD-4 and HD6 have not been observed in these model systems^[11, 22, 23, 24, 25]. In inflammatory bowel disease, reduced expression of hBD-2 and hBD-3 has been described in Crohn's disease, while increases have been found in the colon of ulcerative colitis in the inflamed areas^[26]. Together, a modulating effect of cytokines on the up- or down regulation of defensins in pouchitis can be postulated. The β -defensin hBD-1 expression shows the most striking difference between ulcerative colitis pouches and FAP pouches with strong increases in the non inflamed FAP pouches and relatively low expression in UC pouches. The inverse correlation of hBD-1 and cytokine expression is obvious. Thus, the increased levels of hBD-1 and the low cytokine levels are correlated with low incidence of pouchitis.

Microbials or their products could be involved in defensin regulation in pouches after surgery and in pouchitis. β -defensins are upregulated in response to bacterial antigens or in the presence of cytokines^[22]. Thus, the rise of hBD-2 and hBD-3 in pouchitis reflects mucosal damage and infection. The gene regulatory mechanisms that control hBD-1 expression remain speculative, since hBD-1 was previously classified as being steadily expressed by previous *in vitro* studies^[11]. Fehlbaum *et al.*^[27] described the induction of β -defensin expression by L-isoleucin, which as an essential amino acid must arise from external sources or from degradation of host proteins liberated by bacterial proteases. In this context, L-isoleucin could serve as a marker for microbial presence or for host tissue degradation. Presence of L-isoleucin in turn would then signal to the host the need to increase defence mechanisms.

Faecal stream and faecal stasis in pouches have been postulated to be involved in the pathomechanisms of primary pouchitis^[3, 28]. As long as the faecal stream is diverted from the pouch through a protective ileostomy, pouches do not develop primary pouchitis. In addition, the ileum of the ileostomy does not react with inflammation, although faeces pass through the ileostomy. A variety of pathogenic bacteria have been found in patients with pouchitis, e.g. *Clostridium perfringens*^[29]. A shift from the normal enteral microbial spectrum towards a pronounced presence of anaerobes, a reduction of bacterial diversity and an increase in fungal diversity have been described in ulcerative colitis^[8]. Since each individual defensin has a characteristic pattern of antimicrobial activity, an alteration of defensin expression or a general reduction of defensins is likely to result in marked quantitative and qualitative changes of the luminal microbial contents. Future studies have to focus on the interaction of various protective mucosal factors and the intestinal contents.

In conclusion, the present data correlate the development of pouchitis in ulcerative colitis with changes of defensin expression pattern and cytokine expression. Surgical construction of an ileoanal pouch resulted in massive increases of α - and β - defensin levels in the ileum type pouch mucosa. In general, the expression of defensins in

pouches was significantly higher than in the normal ileum. The initial high levels of α - defensins after pouch surgery in ulcerative colitis appear to be protective for the pouch mucosa, since the decrease of α - defensins upon time correlates with the incidence of pouchitis in ulcerative colitis. Especially for the FAP pouches, hBD-1 could be another protector for the development of pouchitis. Cytokines, metaplastic changes of the ileum mucosa and inappropriate expression of defensin antimicrobial peptides appear to play major roles in the pathomechanism of pouchitis.

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Expression patterns of cytokine, growth factor and cell cycle-related genes after partial hepatectomy in rats with thioacetamide-induced cirrhosis

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Abstract

AIM: To examine the differences in the responses of normal and cirrhotic livers to partial hepatectomy in relation to the factors influencing liver regeneration.

METHODS: Cirrhosis was induced in rats by administration of thioacetamide. Untreated rats were used as controls. The control rats as well as the cirrhotic rats were subjected to 70% partial hepatectomy. At different time points after hepatectomy, the livers were collected and the levels of cytokines, growth factors and cell cycle proteins were analyzed.

RESULTS: After hepatectomy, the cirrhotic remnant expressed significantly lower levels of cyclin D1, its kinase partner, cdk4, and cyclin E as compared to the controls up to 72 h post hepatectomy. Significantly lower levels of cyclin A and cdk2 were also observed while the cdk inhibitor, p27 was significantly higher. In addition, the cirrhotic group had lower IL-6 levels than the control group at all time points up to 72 h following resection.

CONCLUSION: The data from our study shows that impaired liver regeneration in cirrhotic remnants is associated with low expression of cyclins and cdks. This might be the consequence of the low IL-6 levels in cirrhotic liver remnant which would in turn influence the actions of transcription factors that regulate genes involved in cell proliferation and metabolic homeostasis during the regeneration process.

INTRODUCTION

A healthy liver has the capacity to regenerate itself after injury. This results in the restoration of liver mass and function^[1,2]. In the healthy state, hepatocytes are in the resting G0 state and rarely divide. However, following injury or hepatectomy, the process of regeneration is activated. Hepatocytes then undergo transition into the G1 phase, followed by the S phase, G2 phase and mitosis. The passage through the cell cycle is modulated by the interplay between cyclins, cyclin-dependent kinases (cdks) and inhibitors of cdks^[3-6].

Liver regeneration is influenced by a multitude of factors. These include cytokines and growth factors which are necessary to make quiescent hepatocytes replicate^[1,2,7]. It has now been established that a set of priming events is necessary before the normally quiescent hepatocytes can respond to the growth factors. Both tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6) have been shown to be the factors that facilitate the priming events in regeneration^[8,9]. Following the priming events, growth factors then play an important role as mitogens generating a cascade of signals leading to DNA synthesis and cell division^[10].

In humans, surgical resection is often carried out on a diseased liver and is the main therapeutic intervention for hepatocellular carcinoma^[11]. The remnant liver is usually cirrhotic with impaired regenerative capacity. In this study, we examine the differences in the responses of normal and cirrhotic livers to partial hepatectomy in relation to the factors influencing hepatocyte proliferation and division. The thioacetamide-induced rodent model of liver cirrhosis was employed as induction of cirrhosis with thioacetamide is highly reproducible, irreversible and produces morphological features similar to that observed in human cirrhosis^[12].

MATERIALS AND METHODS

Reagents

Mouse monoclonal antibodies against cyclin D1 and cdk2, and rabbit polyclonal antibodies against cyclin A, cyclin D3, cyclin E, cdk4 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Acrylamide, N, N'-methylene-bis-acrylamide, glycine, sodium dodecyl sulfate, 2-mercaptoethanol, isopropyl alcohol and mineral oil were from Sigma (St. Louis, MO, USA). Tween 20 was from Ducheфа (Haarlem, Netherlands). Enhanced chemiluminescence western blotting detection kit, high performance film and nitrocellulose membrane were from Amersham Biosciences (Little Chalfont, Buckinghamshire, England). Sucrose, Bio-Rad protein assay dye, N, N, N', N'-tetra-methylethylenediamine, ammonium persulfate and prestained SDS-PAGE Standards Broad Range were from Bio-Rad (Hercules, CA, USA). Trizol reagent was from GIBCO BRL. Chloroform and sodium chloride were obtained from Merck (Darmstadt, Germany). Access reverse transcription-PCR system was from Promega (Madison, WI, USA). Rat TNF- α and IL-6 ELISA kits were from Bender MedSystems (Vienna, Austria).

Induction of cirrhosis and partial hepatectomy

The induction of cirrhosis, partial hepatectomy (PH) and harvesting of the livers has been previously described^[13,14]. In brief, cirrhosis was induced in ten-week-old male Wistar-Furth rats by intraperitoneal injection of 300 mg thioacetamide/kg thrice weekly for 10 wk. Healthy, control animals were kept under the same conditions without any treatment. After one week acclimation to allow for the washout of thioacetamide, all animals underwent 70% PH as described by Higgins and Anderson^[15]. Two experimental groups were studied. Healthy rats in the control groups as well as cirrhotic rats in the cirrhotic group were submitted to PH. Within each group, six rats were sacrificed at 3, 6, 24, 48 and 72 h after surgery. The excised and the resected livers were harvested and used for further analysis.

Preparation of rat liver lysates

The liver was chopped into small pieces and homogenized in 5 volumes of buffer containing 0.25 mol/L sucrose, 5 mmol/L HEPES and 0.5 mmol/L EGTA, pH 7.5. The homogenate was centrifuged at 12 000 $\times g$ at 4 °C for 10 min. The supernatant (the liver lysate) was then stored in small aliquots at -80 °C till required. The protein concentration was determined using the Bio-Rad protein assay dye reagent with bovine serum albumin as the standard. For each time point, the lysates (containing equal amount of protein from each liver, $n=6$) were pooled and then used for Western blot and ELISA analysis.

Western blotting

25 μg of the pooled sample was resolved on SDS-PAGE and transferred onto nitrocellulose membrane. Mouse monoclonal antibodies against cyclin D1 and cdk2, and rabbit polyclonal antibodies against cyclin A, cyclin D3, cyclin E, cdk4 were used to detect the respective proteins. The bound antibodies were visualized by using enhanced

Table 1 Primers for reverse-transcription-PCR of HGF, TGF- α , p21, p27 and p53 mRNAs and 28S rRNA.

| Target sequence ¹ | Primer | Sequence (5'-3') |
|-------------------------------|-----------------------|-------------------------------|
| 28S | 28S Forward | GGC CAA GCG TTC ATA GCG AC |
| | 28S Reverse | GAG GCG TTC AGT CAT AAT CC |
| HGF ^[38] | HGF Forward | CCC GGT GCT GCA GCA TGT CCT |
| | HGF Reverse | TCC CCT CGA TTT CGA CAG |
| TGF- α ^[38] | TGF- α Forward | GAC AAG TTG AAC AAG AAC CTC |
| | TGF- α Reverse | CGT CAT CCA CCT AAT ACA TAA G |
| p21 ^[52] | p21 Forward | ATG TCC GAT CCT GGT GAT GTC |
| | p21 Reverse | CAC TTC AGG GCT TTC TCT TGC |
| p27 ^[52] | p27 Forward | GCA GCT TGC CCG AGT TCT AC |
| | p27 Reverse | TTC TTG GGC GTC TGC TCC AC |
| p53 ^[53] | p53 Forward | TCC TCC CCA ACA TCT TAT CC |
| | p53 Reverse | GCA CAA ACA CGA ACC TCA AA |

¹The 28 S primers were kindly provided by Assoc. Prof. Chang Chan Fong (Department of Biochemistry, National University of Singapore). The other primers were as previously reported^[38,52,53].

chemiluminescence. The bands were then quantified using the Analytical Imaging Station software (Imaging Research Inc, St. Catharines, Ontario, Canada).

Quantitative assay for TNF- α and IL-6

The concentrations of TNF- α and IL-6 present in the liver lysates were determined using the Rat TNF- α and IL-6 ELISA kits. The assays were carried out according to the protocol described in the manufacturer's manual.

RNA extraction, reverse transcription, and PCR

Total RNA was extracted from the liver samples using the Trizol reagent. The extraction was carried out according to the manufacturer's instructions. For each time point, the total RNA (containing equal amount of RNA from each liver as determined by absorbance at 260 nm, $n=6$) was pooled and then used for reverse transcription-PCR.

Six sets of primers were used for PCR and the sequences are shown in Table 1. The isolated RNA was subjected to reverse transcription-PCR using the Access reverse transcription-PCR system (Promega, USA). A typical 25 μL reverse transcription-PCR reaction contained 0.4 μg of RNA, 0.1 mmol/L dNTPs, 0.5 $\mu mol/L$ of the forward and corresponding reverse primers, 0.5 mmol/L of MgSO₄, and 0.05 U/ μL of AMV reverse transcriptase and 0.05 U/ μL of T7/DNA polymerase. The reverse transcription was carried out at 48 °C for 45 min followed by denaturation at 94 °C for 2 min. Following reverse transcription, the cDNA was subjected directly to PCR. Each cycle consists of denaturation at 94 °C for 30 s, annealing at 60 °C for 1 min and extension at 68 °C for 2 min. At the end of the last cycle, a final extension at 68 °C for 7 min was carried out. The number of PCR cycles used was the lowest needed to produce a product which could be visualized on the gel. The product was separated by agarose-gel electrophoresis and visualized by ethidium-bromide staining. The gel image was captured and the bands were then quantified using the Analytical Imaging Station software (Imaging Research Inc, St. Catharines, Ontario, Canada).

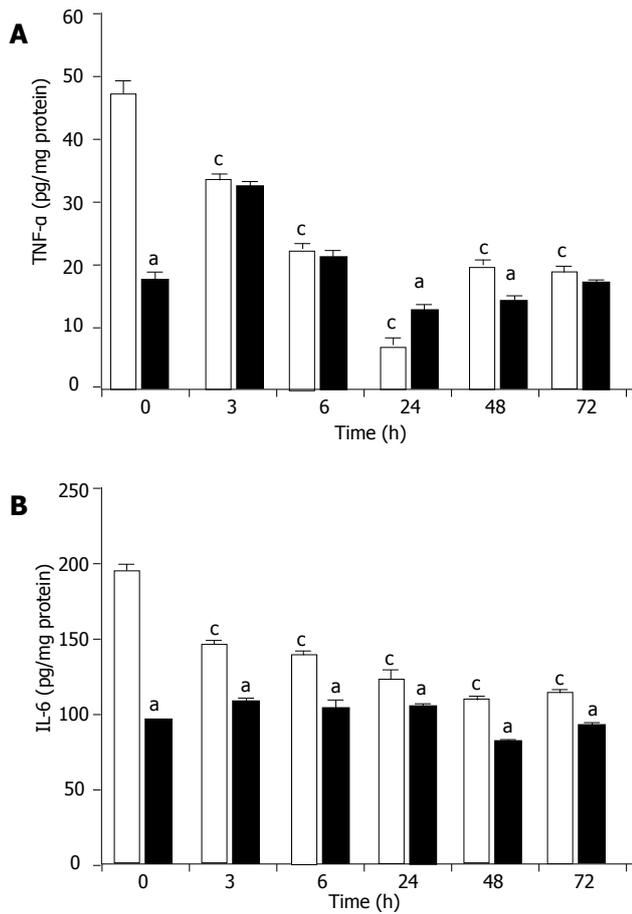


Figure 1 Hepatic **A:** TNF- α and **B:** IL-6 concentrations after PH on healthy rats (white bars) and cirrhotic rats (black bars). At different time points after the operation, the remnant liver was harvested, homogenized and the supernatant was used for analysis. For each time point, the supernatant from 6 animals was pooled and used for ELISA analysis. All analyses were carried out in triplicates and the results are expressed as mean + SD. ^o*P*<0.05, ANOVA analysis, for comparison between control rats before (at 0 h) and after PH. ^a*P*<0.05, ANOVA analysis, for comparison between the cirrhotic group and the corresponding control group at each time point.

RESULTS

Two groups of animals were used in the study. The control group and the cirrhotic group were subjected to 70% PH. All data generated in this study were first examined to determine the changes that occur after PH in the controls. This was followed by comparisons to determine if there were significant differences between the control group and the cirrhotic group both before and after 70% PH.

Hepatic TNF- α and IL-6

Prior to PH, TNF- α and IL-6 were both detected in the liver extracts from the control group and the cirrhotic group. The levels in cirrhotic livers were significantly lower than that in healthy livers. Following resection, the hepatic levels of both TNF- α and IL-6 were significantly lowered from 3 h till 72 h post-resection in the control group. Significantly different TNF- α levels between the controls and cirrhotics were only observed at 24 h and 48 h post-PH. Reduced IL-6 levels were observed in the cirrhotic group before and up to 72 h following PH (Figure 1A and B).

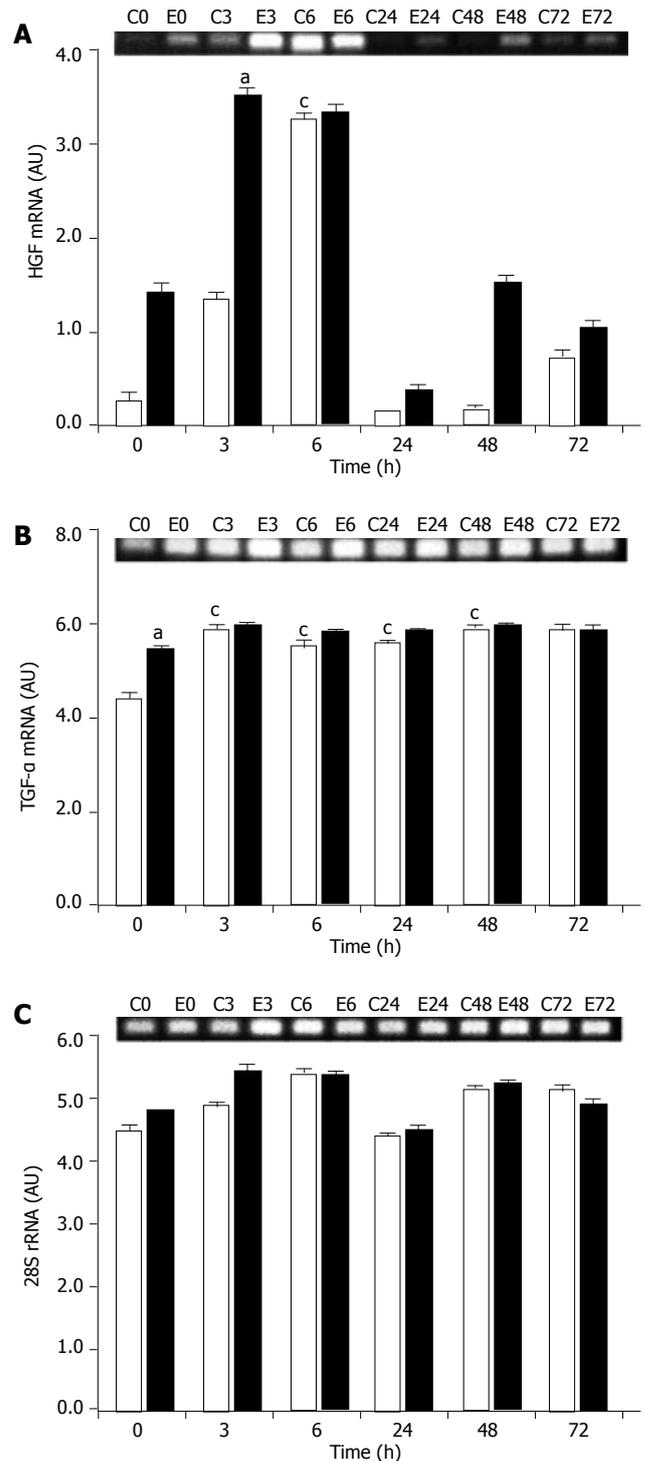


Figure 2 Hepatic **A:** HGF, **B:** TGF- α and **C:** 28S rRNA expressions after PH on healthy rats (white bars) and cirrhotic rats (black bars). At different time points after the operation, the remnant liver was harvested, and RNA was extracted. For each time point, the RNA from 6 animals was pooled and used for reverse-transcription followed by PCR. The PCR product was separated by agarose-gel electrophoresis and visualized by ethidium-bromide staining. The gel image was captured and the bands were quantified using the Analytical Imaging Station software. All analyses were carried out in triplicates. A representative gel (C, control, healthy rats; E, cirrhotic rats; the number indicates the time post-PH in h) and the quantification analysis expressed as mean + SE (AU = arbitrary units) are shown. ^o*P*<0.05, ANOVA analysis, for comparison between control rats before (at 0 h) and after PH. ^a*P*<0.05, ANOVA analysis, for comparison between the cirrhotic group and the corresponding control group at each time point.

Expression of growth factors

Total RNA was extracted from the liver samples and sub-

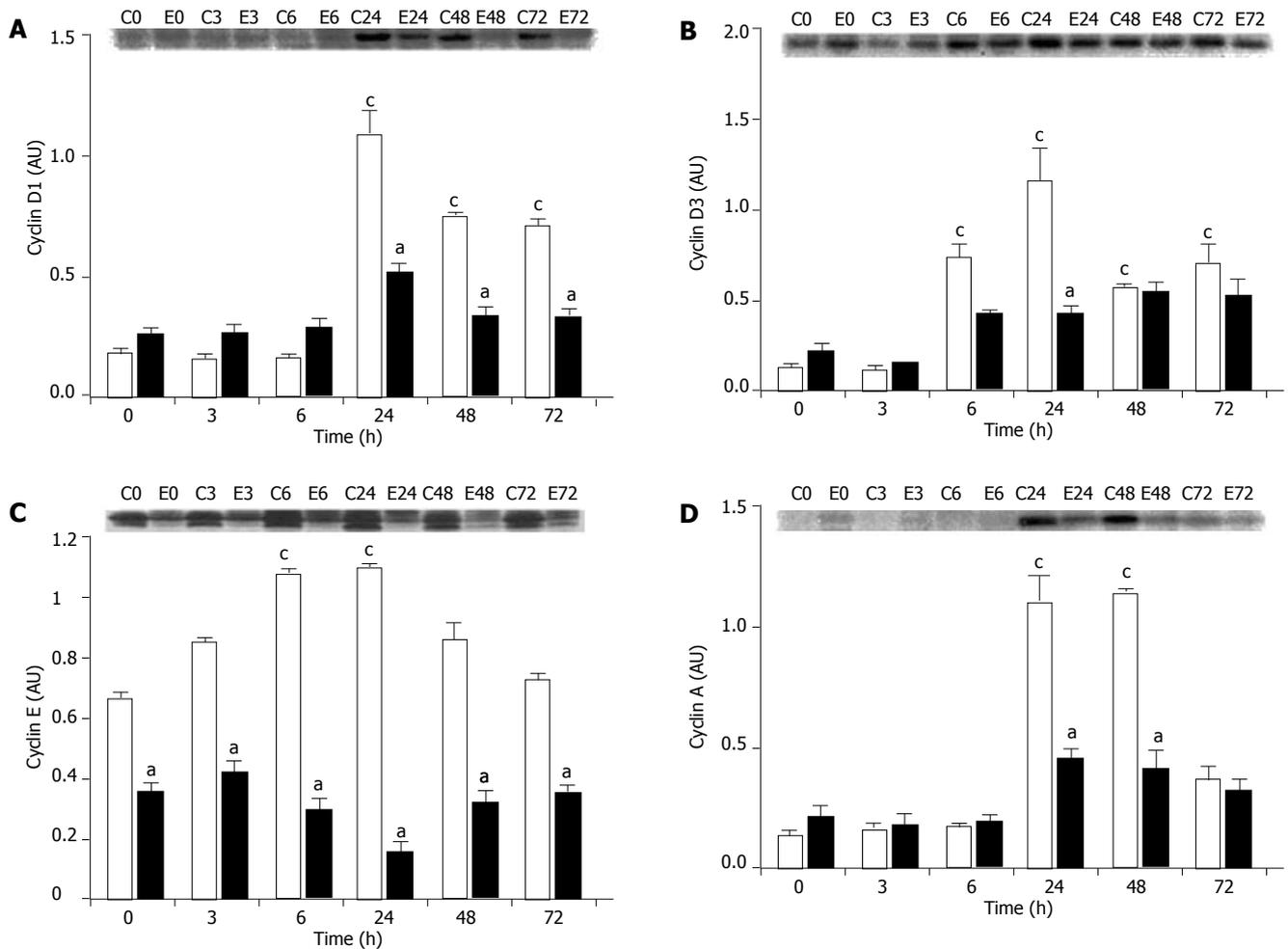


Figure 3 Effect of PH on cyclin expressions in healthy rats (white bars) and cirrhotic rats (black bars). At different time points after the operation, the remnant liver was harvested, homogenized and the supernatant was used for analysis. For each time point, the supernatant from 6 animals was pooled and used for Western blot analysis. The blot image was captured and the bands were quantified using the Analytical Imaging Station software. All analyses were carried out in triplicates. A representative blot (C, control, healthy rats; E, cirrhotic rats; the number indicates the time post-PH in h) and the quantification analysis expressed as mean \pm SE (AU = arbitrary units) are shown. **A:** Cyclin D1 expression. **B:** Cyclin D3 expression. **C:** Cyclin E expression. **D:** Cyclin A expression. $^{\circ}P < 0.05$, ANOVA analysis, for comparison between control rats before (at 0 h) and after PH. $^{\circ}P < 0.05$, ANOVA analysis, for comparison between the cirrhotic group and the corresponding control group at each time point.

jected to reverse transcription-PCR analysis for transcripts encoding for the hepatocyte growth factor (HGF) and the transforming growth factor alpha (TGF- α). A transient increase in HGF was observed at 6 h post-PH for the control group. The trend was also similar for the cirrhotic group, although a significant increase was also observed at 3 h post-PH (Figure 2A).

In contrast, TGF- α was significantly higher at all time points up till 48 h post-resection in the control group. TGF- α level in the cirrhotic livers was significantly higher than controls before PH. After PH, the levels remained high and there were no differences between the control group and the cirrhotic group (Figure 2B). The 28S rRNA was used as a control and no significant differences in the expression were observed (Figure 2C).

Markers of cell cycle progression, cdk inhibitors and p53 expression

Western blot analysis was carried out to examine the changes in cyclins and cdk. In the control group, cyclin D1 levels were elevated from 24 to 72 h post-PH (Figure 3A) while cyclin D3 levels peaked at 24 h post-PH and

remained significantly elevated up till 72 h post-PH (Figure 3B). In addition, cdk4 was also significantly increased at the 6 h time point (Figure 4A). Comparison of the cirrhotic group with the corresponding controls showed that cyclin D1 levels were significantly lower at 24 to 72 h post-PH and the peak in cyclin D3 expression was not observed. The cdk4 levels were also lower than that of the corresponding controls from 6 to 72 h post-resection (Figure 3A, 3B and 4A).

As previously described, the antibody against cyclin E recognized multiple isoforms^[16,17]. These represent alternative spliced variants of the gene. Cyclin E levels in the controls increased significantly within 24 h following resection. However, in the cirrhotic group, this increase was not evident and cyclin E levels were lower than the controls at all time points (Figure 3C). For the control animals, the highest levels of cyclin A and cdk2 expression were at 24 and 48 h after resection. At these time points, the levels of cyclin A and cdk2 in the cirrhotic animals were significantly lower than that in the corresponding controls (Figure 3D and 4B).

Reverse transcription-PCR analysis showed that for

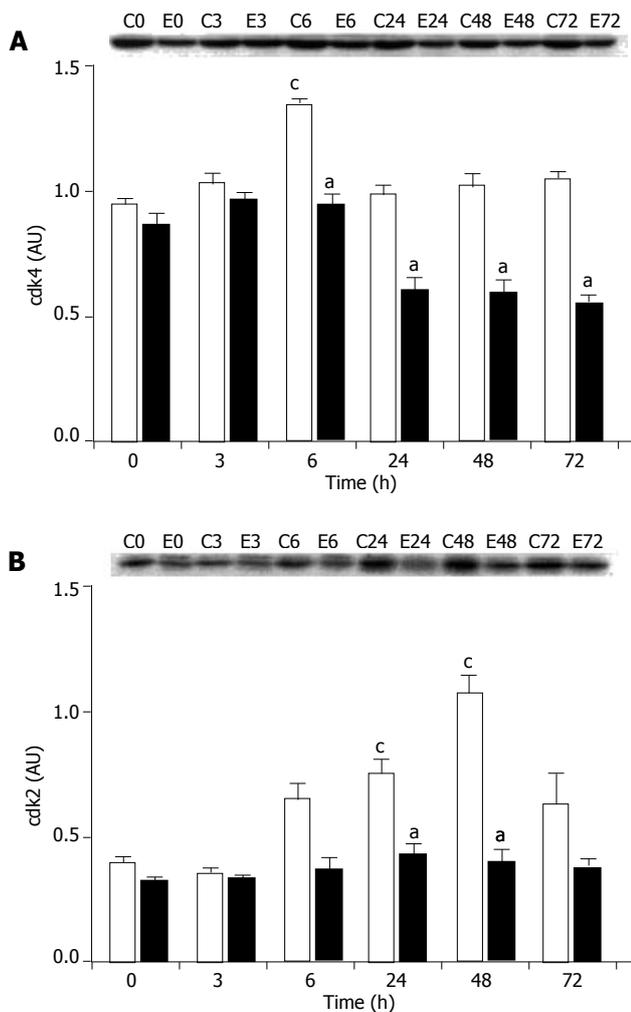


Figure 4 Effect of PH on cyclin-dependent kinase expressions in healthy rats (white bars) and cirrhotic rats (black bars). At different time points after the operation, the remnant liver was harvested, homogenized and the supernatant was used for analysis. For each time point, the supernatant from 6 animals was pooled and used for Western blot analysis. The blot image was captured and the bands were quantified using the Analytical Imaging Station software. All analyses were carried out in triplicates. A representative blot (C, control, healthy rats; E, cirrhotic rats; the number indicates the time post-PH in h) and the quantification analysis expressed as mean+SE (AU = arbitrary units) are shown. **A:** Expression of cdk4. **B:** Expression of cdk2. ^c $P < 0.05$, ANOVA analysis, for comparison between control rats before (at 0 h) and after PH. ^a $P < 0.05$, ANOVA analysis, for comparison between the cirrhotic group and the corresponding control group at each time point.

both p21 and p53, the increase in expression was before the 24 h time point for the control group and the cirrhotic group (Figure 5A and C). However, the expression of p27 in the cirrhotic group was significantly higher than the controls before as well as after resection (Figure 5B).

DISCUSSION

In this study, the thioacetamide-induced rodent model of liver cirrhosis was used to examine the factors that may contribute to impaired regeneration of cirrhotic livers after PH. Changes in the expression patterns of cytokines, growth factors and cell cycle proteins were examined over a period of 72 h.

Cyclins, cdks and cdk inhibitors were all detected in healthy livers, similar to previous observations^[16]. Cirrhotic

livers also expressed comparable levels of D-type cyclins, cyclin A, cdks, p21 and p53 as the healthy livers. However, cyclin E was significantly lowered while p27 was significantly raised.

Following PH, quiescent hepatocytes are induced to proliferate. Cyclins and their kinase partners play key roles in regulating the progress through the different phases of the cell cycle^[18,19]. The D-type cyclins together with their kinase partners play a key role in regulating G1 progression^[20]. The cyclinE/cdk2 complex regulates G1 to S phase transition^[21], while the cyclinA/cdk2 complex is necessary for initiation of DNA replication in the S phase^[22-24]. As expected, the expression of cyclin D1, cyclin E and cyclin A increased significantly in the controls after resection as the liver undergoes regeneration. This is congruent with other studies which showed that after PH, there is firstly an induction of G1 cyclins preceding S phase followed by subsequent induction of cyclin A and cyclin B^[4,25,26].

Transient increases in the mRNA levels of cdk inhibitors p21 and p27, as well as p53 were also observed at 3-6 h after PH in the controls. Similar observations have been previously described in various other studies using rodent models^[6, 27-30]. It is thought that in the regenerating liver, p53 plays a dual role^[30]. The first is as a cell cycle check resulting in a delay in the G1 phase to allow damage repair^[29]. The second is the induction of the expression of growth factors or their receptors. Indeed, p53 responsive elements have been found on the promoters of HGF and TGF- α , both of which are involved in the proliferative response^[31,32]. The expression of the cdk inhibitor, p21 is also induced post PH, probably in part as a response to p53 induction^[33]. This may serve to prevent premature progression through G1 phase^[34] or to stabilize the cyclinD-cdk4 complex and promote its translocation to the nucleus^[35]. p27 acts as a brake on cyclinE-cdk2 activity and has to be sequestered by cyclin D1 for cdk2 to be active^[36].

Comparison between the control and cirrhotic groups showed that the changes in cyclin/cdk expressions after PH were not mirrored in the cirrhotic livers. Cyclin D1 and its kinase partner, cdk4 were expressed at much lower levels at 24-72 h post PH, cyclin A and cdk2 were decreased at 24-48 h post PH while cyclin E was persistently lowered up to 72 h post PH. In addition, the expression of the cdk inhibitory protein, p27 was also elevated at 6-48 h post PH. Thus, unlike healthy livers, cirrhotic livers expresses significantly lower levels of cyclins [this study and those by Zhao *et al.*^[37] and Masson *et al.*^[38]]. Given the persistent low levels of G1 cyclins, it is likely that the block to cell cycle progression occurs at the G1 phase.

The key factors that influence liver regeneration include the cytokines TNF- α and IL-6 and the growth factors HGF and TGF- α . The cytokines TNF- α and IL6, are necessary to prime and initiate liver regeneration after PH^[28,9]. Signals to initiate liver regeneration for TNF- α are mediated by TNF receptor 1. Knockout mice lacking this receptor have diminished ability to restore liver mass after PH leading to increased mortality^[39]. High postoperative mortality and impaired response to PH were also observed in IL-6 deficient mice^[40]. Besides the cytokines, growth factors also play important roles in liver regeneration. TGF- α and HGF are potent mitogens for hepatocytes^[10]. The re-

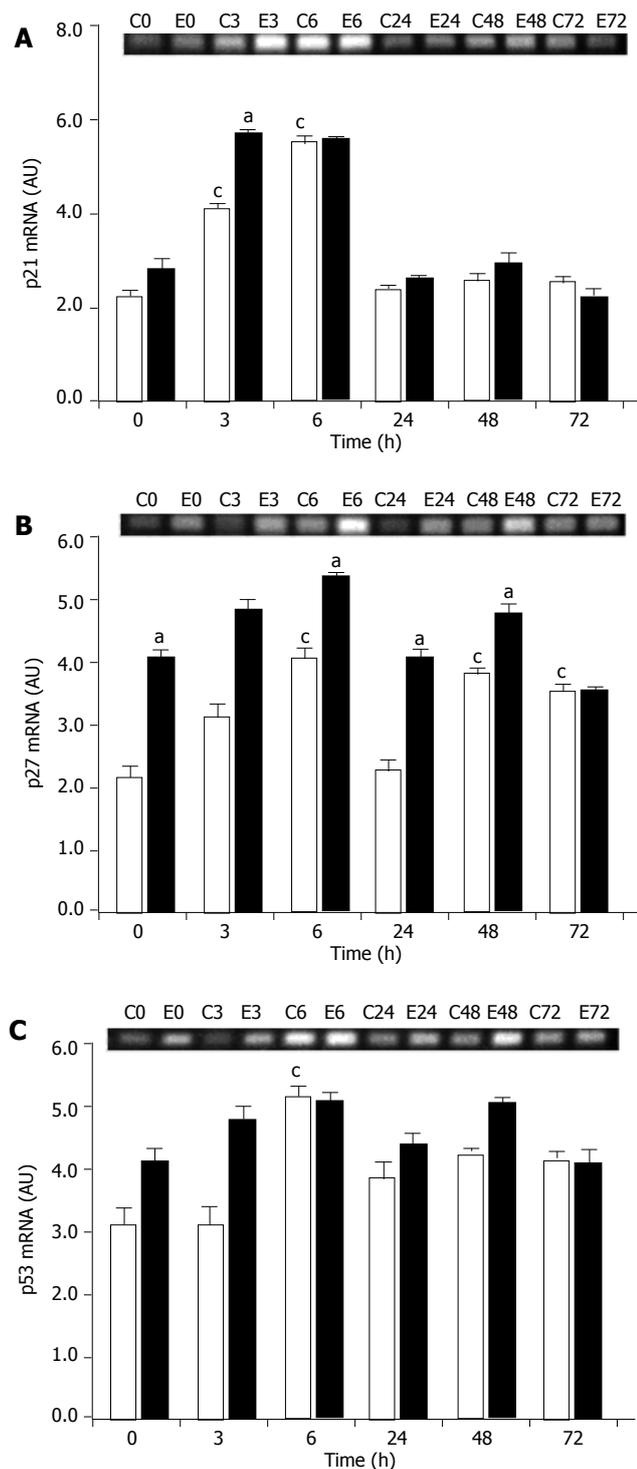


Figure 5 Hepatic **A**: p21, **B**: p27 and **C**: p53 expressions after PH on healthy rats (white bars) and cirrhotic rats (black bars). At different time points after the operation, the remnant liver was harvested, and RNA was extracted. For each time point, the RNA from 6 animals was pooled and used for reverse-transcription followed by PCR. The PCR product was separated by agarose-gel electrophoresis and visualized by ethidium-bromide staining. The gel image was captured and the bands were quantified using the Analytical Imaging Station software. All analyses were carried out in triplicates. A representative gel (C, control, healthy rats; E, cirrhotic rats; the number indicates the time post-PH in h) and the quantification analysis expressed as mean + SE (AU = arbitrary units) are shown. ^c $P < 0.05$, ANOVA analysis, for comparison between control rats before (at 0 h) and after PH. ^a $P < 0.05$, ANOVA analysis, for comparison between the cirrhotic group and the corresponding control group at each time point.

sponse to HGF is mediated by its interaction with c-met, a protooncogene which encodes a receptor tyrosine kinase.

In conditional met mutant mice, impaired regeneration occurs after PH and this is due to a defective exit from quiescence and diminished entry into S phase^[41]. In addition, HGF also upregulates the expression of TGF- α and the presence of anti-TGF α antibodies can block DNA synthesis in regenerating livers^[42].

Cirrhotic livers have significantly lower TNF- α and IL-6 levels compared to healthy livers prior to PH. It is unclear why this should be so. However, similar reductions have been observed in patients with primary biliary cirrhosis and fulminant hepatitis^[43,44]. This is not simply due to a general depression in RNA synthesis in the diseased livers as the expression of other genes such as IL-1 and interferons were unaffected^[44]. In addition, unchecked fibrosis will lead to impaired diffusion of nutrient and hepatotropic factors and this may also be a contributing factor to the reduced levels of TNF- α and IL-6. In contrast, HGF expression was unchanged in cirrhotic livers. TGF- α expression was significantly increased in cirrhotic livers and this is consistent with the role of TGF- α in the chronic regeneration that occurs in the cirrhotic process^[45].

Following resection of healthy controls, it was observed that both TNF- α and IL-6 levels in the liver decreased progressively over a period of 72 h. This is to be expected as TNF- α and IL-6 are necessary mainly for the initial priming events. In addition, TNF- α is a pro-inflammatory cytokine and high levels are not desirable once the priming signals have been initiated. In this study, a peak in both cytokines was not observed after PH. This is probably due to the fact that the increase in both cytokines was expected to be at an early time point after resection^[39,46] and the first time point in this study may be too late for this observation. A peak in the expression of HGF was observed at 3-6 h post PH while that of TGF- α was elevated up to 48 h post PH. Similar to that observed by Zhao *et al*, hepatic expression of the growth factors HGF and TGF- α was not significantly different in both normal and cirrhotic livers after PH^[37].

In contrast to the growth factors, it was observed that following partial hepatectomy, the IL-6 levels in cirrhotic livers are significantly lower than that of the healthy controls. The role of IL-6 in liver regeneration has been extensively examined. To date, the roles of IL-6 in liver regeneration include the activation of acute phase response, induction of proliferation and hepatoprotection. The binding of IL-6 to the gp130-IL-6 receptor complex results in the activation of Janus kinase (JAK). This in turn activates the mitogen-activated protein kinase (MAPK) and STAT3 signaling pathways^[7,9]. In IL-6 deficient mice, liver failure and defective hepatocyte regeneration is observed following PH. Subcutaneous injection of IL-6 which results in sustained action of IL-6 was able to completely reverse the high post-operative mortality of IL-6 deficient mice^[47]. It is interesting to note that both IL-6 and cyclin D1 levels were reduced in the regenerating cirrhotic liver. AP1 activity was also markedly inhibited in regenerating cirrhotic livers with reduced expression levels of c-Jun and JunD^[37]. Taken together, these observations are comparable to that in IL-6 deficient mice which shows marked reduction in STAT3 activation and depressed AP-1, myc and cyclin-D1 expressions^[40].

IL-6 also mediates the induction of C/EBP expression^[48]. Impaired C/EBP β activities have been observed in cirrhotic livers following resection^[37] and this could account for the reduction in cyclin E and cyclin A. In C/EBP β -/- mice, cyclins A, B and D were reduced near the peak of DNA synthesis during liver regeneration^[49]. Although it is still uncertain as to whether C/EBP β regulates cyclin expression via transcriptional or post-transcriptional mechanisms, it is clear that in C/EBP β -/- hepatocytes, cell cycle progression is blocked close to or at the G1/S phase transition^[49].

The data from our study thus shows that impaired liver regeneration in cirrhotic remnants is associated with low expression of cyclins including cyclin D1. In rats with carbon tetrachloride-induced cirrhosis, similar changes in the expression of cyclins and IL-6 were also observed^[38]. Markedly reduced IL-6 levels could influence the action of transcription factors (such as STAT3, AP1, C/EBP β and HNF-1 α) which are necessary for the regulation of genes that coordinate the balance between cell proliferation and metabolic homeostasis during liver repair and growth. In addition, liver regeneration is also critically dependent on ATP stores and the reduction in liver proliferation in cirrhotic livers may be due in part to inadequate mitochondrial respiratory function. Cirrhotic livers indeed have reduced complex I activity and lower respiratory control ratios following PH as shown by us and others^[13,50,51].

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***Eubacterium limosum* ameliorates experimental colitis and metabolite of microbe attenuates colonic inflammatory action with increase of mucosal integrity**

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Abstract

AIM: To examine the effect of *Eubacterium limosum* (*E. limosum*) on colonic epithelial cell line *in vitro*, and to evaluate the effect of *E. limosum* on experimental colitis.

METHODS: *E. limosum* was inoculated anaerobically and its metabolites were obtained. The growth stimulatory effect of the *E. limosum* metabolites on T84 cells was evaluated by SUDH activity, and the anti-inflammatory effect by IL-6 production. The change in mRNA of toll like receptor 4 (TLR4) was evaluated by real time PCR. Colitis was induced by feeding BALB/C mice with 2.0% dextran sodium sulfate. These mice received either 5% lyophilized *E. limosum* ($n = 7$) or control diet ($n = 7$). Seven days after colitis induction, clinical and histological scores, colon length, and cecal organic acid levels were determined.

RESULTS: The *E. limosum* produced butyrate, acetate, propionate, and lactate at 0.25, 1.0, 0.025 and 0.07 mmol/L, respectively in medium. At this concentration, each acid had no growth stimulating activity on T84 cells; however, when these acids were mixed together at the above levels, it showed significantly high activity than control. Except for lactate, these acids significantly attenuated IL-6 production at just 0.1 mmol/L. In addition, under TNF- α stimulation, butyrate attenuated the production of TLR4 mRNA. The treatment with *E. limosum* significantly attenuated clinical and

histological scores of colitis with an increase of cecal butyrate levels, compared with the control group.

CONCLUSION: *E. limosum* can ameliorate experimental colonic inflammation. In part, the metabolite of *E. limosum*, butyrate, increases mucosal integrity and shows anti-inflammatory action modulation of mucosal defense system *via* TLR4.

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Key words: Intestinal Microflora; Butyrate; *Eubacterium limosum*; Toll like receptor; IL-6

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INTRODUCTION

Inflammatory bowel disease (IBD), which includes ulcerative colitis (UC) and Crohn's disease, is a chronic and aggressive idiopathic inflammatory disorder of the gastrointestinal tract.^[1] The detailed mechanism and causes of IBD are not fully understood; however, abundant experimental observations suggest that luminal bacteria (microflora) play a crucial role in the pathogenesis of IBD.^[2,3,4] In experimental colitis models, colitis has not occurred without the presence of intestinal flora. This indicates that microflora are likely to induce colonic inflammation and its recurrence.^[5] Recently, therapeutic approaches to modifying microflora have been attempted by the use of antibiotics, probiotics, and prebiotics.^[4,6,7]

Germinated barley foodstuff (GBF), which consists mainly of dietary fiber and glutamine (Gln)-rich protein, is a prebiotic product for UC patients.^[8] In our previous studies, GBF exhibited preventive and therapeutic effects in experimental colitis models, especially an improvement of severe bloody diarrhea and attenuation of colonic mucosal damage, accompanied by the modulation of microflora.^[9] In colitis models, GBF or its fiber fraction

significantly increased cecal butyrate production, and GBF significantly increased the number of butyrate producing *Eubacterium limosum* (*E. limosum*), with a decrease in colonic pH.^[9,10] As a result, in this colitis model, GBF was considered to be efficiently fermented and converted by *E. limosum* into short-chain fatty acids (SCFAs), including butyrate.^[8,9,11]

Furthermore, a clinical trial of GBF showed that this treatment can benefit UC patients by increasing the level of stool butyrate. This clinical efficacy was also associated with an increase in the number of *Bifidobacterium* and *E. limosum*, both of which are regarded as beneficial bacteria, in the stool.^[12,13] However, the mechanism of the therapeutic effect of GBF and *E. limosum* has been incompletely understood. The detailed metabolism of *E. limosum* in gastrointestinal tract is also little understood, differing from *Lactobacillus* or *Bifidobacterium* as representative probiotics.^[8] The net viability in human gastrointestinal tract and resistance to gastric or bile acids were not clear in detail, and these difficulties might be caused by the obligate anaerobic character. The only distinct effect of *E. limosum* is increase of butyrate production in ulcerative colitis^[14] and experimental colitis.^[12] Thereupon, we pointed out the major metabolites of *E. limosum* (short chain fatty acids including butyrate) and the minute role in colonic epithelium. Although this study is preliminary, we focus on the role of *E. limosum* *in vivo* and its metabolites on colonic epithelium *in vitro*.

MATERIALS AND METHODS

Preparation of *E. limosum*^[11]

E. limosum (JCM6421 Riken Bioresource Center, Saitama, Japan) was inoculated with modified GAM medium (Nissui Pharmaceutical Co., Tokyo, Japan) anaerobically at 37 °C. AnaeroPack (Mitsubishi Gas Chemical Co., Tokyo, Japan) was used to obtain the anaerobic condition. The *E. limosum* and supernatant were separated by centrifugation and filtration, sequentially lyophilized. The number of *E. limosum* after lyophilization was of the same order as before being cultured (10⁹ CFU/1 g). The organic acid content of the supernatant (residual medium) was determined by HPLC using the method described in our previous study.^[9] Briefly, the supernatant was filtered through a membrane filter and separated with an ion-exclusion column (Shodex Rspak KC-811, 8 mmID × 30 cm long, Showa Denko Co., Ltd., Tokyo, Japan). Organic acids were detected using bromothymol blue as the post-column reagent. The column temperature was 60 °C, and the mobile phase was a 0.75 mmol/L sulfuric acid solution (flow rate : 0.8 mL/min).

Cell culture

The methods for maintenance of T84 (ATCC CCL-248, Rockville, MD) cells in culture were as previously described in other studies.^[15,16] Briefly, T84 cells were grown in monolayers in a 1:1 mixture of Dulbecco's modified Eagle's Medium/Nutrient Mixture F-12 Ham (Sigma St. Louis MO, USA) supplemented with, 50 U/mL penicillin, 50 µg/mL streptomycin and 5% fetal bovine serum. Confluent monolayers were subcultured by trypsinization

with 0.5% trypsin and 5.3 mmol/L EDTA in Ca²⁺ and Mg²⁺ free phosphate-buffered saline.

Growth activity and IL-6 production

For the determination of cell growth activity, succinate dehydrogenase (SUDH) activity was evaluated. Since SUDH was reported to be well related to the proliferative and viability activity^[17], 6 × 10⁴ cells were plated onto each well of 96-well plates and the cells were treated with the respective mediums for 24 h, Cell Counting Kit-8 (Dojin Chemicals Co., Ltd. Kumamoto Japan) was used, then SUDH activity was measured as per the manufacturer's instructions.^[17] For the determination of IL-6, cells were plated onto each well of 48-well plates. The cells were grown to confluence in cell culture dishes. They were treated with TNF-alpha (50ng/mL; Funakoshi Co., Tokyo, Japan) for another 24 h and the supernatants were harvested for measurement of IL-6, IL-6 ELISA (Genzyme Cambridge MA) as per the manufacturer's instructions.

Expression of mRNA level of toll like receptor 4^[18]

For the determination of expression of TLR4 mRNA, T84 cells were plated into each well of 60mm × 15mm cell culture dishes. The cells were grown to confluence in cell culture dishes. They were treated with TNF-alpha (25ng/mL; Funakoshi Co., Tokyo, Japan) with sodium butyrate (0, 0.5, 1.0, and 2.0 mmol/L) for another 6 hrs. Then total RNA extraction was carried out by RNeasy Mini Kit (QIAGEN-JAPAN KK Tokyo) and RT-PCR was performed (Applied Biosystems 7300 real time PCR system, Applied Biosystem-Japan Tokyo Japan). RT-PCR was carried out with specific primers and probes for TLR4 receptor components and housekeeping gene, beta-actin (Taqman gene expression assay, Applied Biosystem-Japan Tokyo Japan). The primer pairs used for amplification of TLR4 were (5'-3') CAG AGT TTC CTG CAA TGG ATC A and GCT TAT CTG AAG GTG TTG CAC AT. TaqMan MGB probes (labeled with fluorescent reporter dye 6FAM) were used for analysis of TLR4 (CGT TCA ACT TCC ACC AAG AGC TGC CT). Respective data were shown after normalized to housekeeping gene (beta-actin).

Animals and diets

Fourteen 8-wk-old female BALB/c mice were purchased from Charles River Japan (Kanagawa, Japan). The mice were housed individually in cages in a room kept at 20 to 25 °C and 40 to 70% relative humidity with a 12 h lighting cycle (conventional conditions).^[12] They were allowed free access to diet and drinking water. First, the 14 mice were fed laboratory chow for 1 wk during their acclimatization period. They were then divided into two groups of 7: a 5% *E. limosum* group (*E. limosum* group) and a cellulose-diet control group (control group). The total volume of protein and dietary fiber in both diets was adjusted to 14.6% protein and 3.0 % dietary fiber.^[19,12] The compositions of the two diets are shown in Table 1.

After one week of pre-feeding, the mice weighing 20 to 22 g in the two groups were given 2% dextran sodium sulfate (mol wt, 5000; Wako Pure Chemical Co., Osaka, Japan) in their diet to induce colitis. The animals were

Table 1 Composition of experimental diets

| | CE | <i>E. limosum</i> (g/kg diet) |
|-------------------|---------|----------------------------------|
| Casein | 146.0 | 100.0 |
| Vitamin mixture | 10.0 | 10.0 |
| Mineral mixture | 35.0 | 35.0 |
| Choline chloride | 2.0 | 2.0 |
| Cellulose | 30.0 | |
| <i>E. limosum</i> | | 50.0 |
| Corn oil | 50.0 | 50.0 |
| DSS | 20.0 | 20.0 |
| Corn starch | 707.0 | 733.0 |
| (without DSS) | (727.0) | (753.0) |

CE; Cellulose

E. limosum (3×10^{12} cells/g)

sacrificed at d 7, and the colon and cecal contents were removed. This animal experiment was approved by the local ethical committee.

Evaluation of disease activity

In all the mice, the body weight, occult blood, and rectal bleeding, as well as stool consistency were monitored daily. The presence of blood in feces was evaluated using a hemocult test kit (Beckman Coulter, Fullerton, CA). The disease activity index was determined by scoring changes in weight, hemocult positivity, gross bleeding, and stool consistency, described in our previous study.¹²⁾ Briefly, we used five grades of weight loss (0: no loss or weight gain, 1: 1 to 5%, 2: 5 to 10%, 3: 10 to 20%, 4: loss of more than 20%), three grades of stool consistency (0: normal, 2: loose, 4: diarrhea) and three grades of occult blood (0: normal, 2: occult blood positive, 4: gross bleeding). And disease activity score was sum of above parameters.

Histological evaluation

The entire colon was removed, and its length was measured. After removal, the colon was immediately divided into three segments (proximal: 1cm from ileo-cecal valve, middle, and distal: 1.5cm from anal columns), and a part of each segment was fixed in 10% neutral buffered formalin. After fixation, the specimens were stained with hematoxylin and eosin.¹²⁾ Six sections from each segment were scored for colitis severity (crypt scores) based on the method of Cooper *et al* by two blind examiners.¹²⁾ Briefly, this scoring method is: grade 0, intact crypt; grade 1, cystic dilatation of crypts; grade 2, loss of the basal one-third of the crypt; grade 3, loss of the basal two-thirds of the crypt; grade 4, loss of entire crypts with the surface epithelium remaining intact; grade 5, loss of both the entire crypt and surface epithelium.^{10,12)} The average damage score was shown in Figure 5.

Cecal organic acid determination

The cecal contents were removed immediately after the dissection. Because the cecal contents of the mice were very small, we had to gather all the samples in the two groups. Organic acids were measured by the HPLC methods described in Experiment 1.⁹⁾

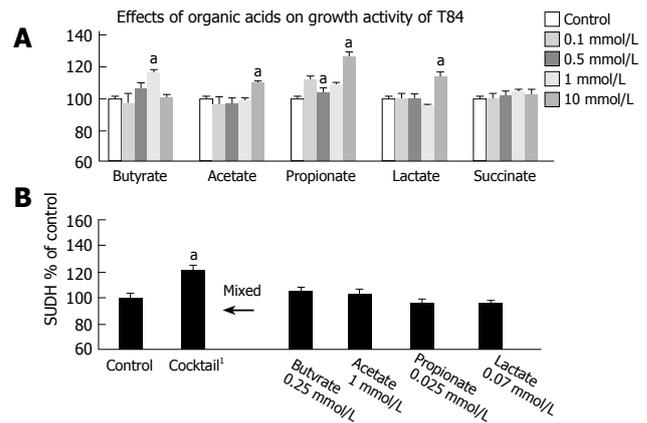


Figure 1 Effect of organic acids (A) and the cocktail mixture of organic acids (B) on SUDH activity in T84 cells. Cells were incubated with various concentrations of fatty acids for 24 hours. The data is expressed as means \pm SD ($n=4$). ^a $P < 0.05$ vs control. ¹The cocktail was an organic acid mixture adjusted to that of 10% *E. limosum* supernatant (butyrate [0.25 mmol/L], acetate [1 mmol/L], propionate [0.025 mmol/L], and lactate [0.07 mmol/L]).

Statistical analysis

Results are expressed as means \pm SD. Differences in the disease activity were evaluated by chi-square analysis. The Student *t* test (two-tailed) was used to test all other comparisons. Significance was accepted at $P < 0.05$.

RESULTS

The effects of organic acids on growth activity of T84 are shown in Figure 1A and B. Figure 1A shows the effects of sodium-butyrate, sodium-acetate, sodium-propionate, sodium-lactate, and sodium-succinate at different levels (0, 0.1 mmol/L, 0.5 mmol/L, 1 mmol/L, and 10 mmol/L) on the growth activity of T84 as determined by SUDH activity. Butyrate significantly increased the growth activity of T84 at 1 mmol/L. Acetate and lactate promoted a significantly high growth activity at just 10 mmol/L. With regards to the propionate, at 0.1 and 10 mmol/L, significant increases were observed. There were no statistical differences in succinate supplementation at all dose levels.

In our previous study, *E. limosum* produced butyrate (2.5 mmol/L), acetate (10 mmol/L), propionate (0.25 mmol/L), and lactate (0.7 mmol/L) in the medium and the addition of 10% *E. limosum* supernatant to T84 significantly increased SUDH activity.¹²⁾ Therefore, the organic acid mixture adjusted to that of 10% of *E. limosum* supernatant (butyrate (0.25 mmol/L), acetate (1 mmol/L), propionate (0.025 mmol/L), and lactate (0.07 mmol/L) was simultaneously added as a cocktail to T84. This cocktail significantly increased the growth activity, as shown in Figure 1 B.

Three kinds of short-chain fatty acids (butyrate, acetate, propionate) promoted a significant decrease of IL-6 production in a dose-dependent manner. Lactate only showed a decrease of IL-6 production at 0.1 mmol/L, and succinate had no anti-inflammatory effects at any dose level. (Figure 2). In this experiment, the cocktail also significantly attenuated IL-6 production (77% of control, $P < 0.05$). However, it does not seem the synergistic effects as in growth stimulatory effect.

Figure 3 showed the effect of butyrate on expression

Table 2 Organic acid analysis of pooled cecal content in colitis model

| | Succinate | Lactate | Acetate | Propionate | n-Butyrate |
|----|--|-------------------|--------------------|-------------------|-------------------|
| | ($\mu\text{mol} / \text{g content}$) | | | | |
| CE | 0.689 \pm 0.572 | 3.211 \pm 2.373 | 17.105 \pm 9.653 | 2.144 \pm 0.881 | 1.644 \pm 0.294 |
| EL | 1.624 \pm 0.439 | 7.445 \pm 2.882 | 38.106 \pm 4.258 | 7.168 \pm 1.000 | 2.523 \pm 0.552 |

Values are the means \pm SD of pooled samples analyzed three times.

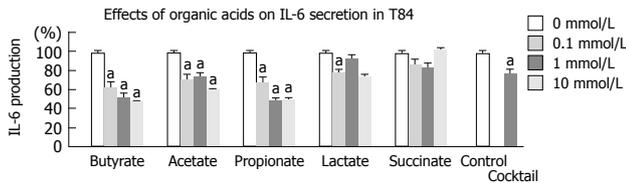


Figure 2 Effect of organic acids on secretion of IL-6 by TNF-alpha stimulation in T84 cells. Cells were incubated with TNF-alpha (50 ng/mL) and various concentrations of organic acids for 24 hours, then supernatant was applied for determination of IL-6 production by ELISA. Data is expressed as means \pm SD ($n=3$). ^a $P<0.05$ vs control.

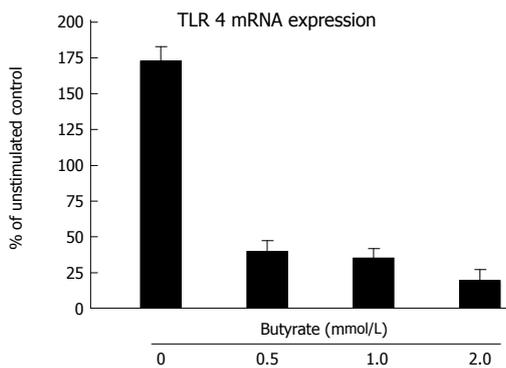


Figure 3 Inhibition of response to TNF -alpha (25 ng/mL) induced the mRNA level of toll-like receptor 4 (TLR4) in T84 by exposure to butyrate. Cells were treated with 0 to 2.0 mmol/L sodium butyrate for 6 hr prior to extraction of RNA and real time PCR (RT-PCR). RT-PCR was performed with specific primers and probs for indicated receptor components and housekeeping gene beta-actin. All data were normalized to the expression of beta-actin, and presented as % value of control (without TNF-alpha treatment). Data is expressed as means \pm SD.

of TLR4 mRNA in T84. Interestingly, butyrate attenuated the expression of TLR4 mRNA at low-level 0.5 mmol/L.

The change in body weight after initiation of colitis is shown in Figure 4. The *E.limosum* group showed a significant retention of body weight on d 6 and 7, compared with the control group. The change in disease activity of both dietary groups is shown in Figure 5. Prior to the change in body weight, the disease activity on d 4 was significantly attenuated in the *E.limosum* group. The organic acid contents in both dietary groups are shown in Table 2. This data is for pooled samples ($n=7$ in respective groups), so statistical analysis was not possible; however, the level of each organic acid dramatically increased.

Figure 6 A shows representative histological photographs of colonic mucosa in both groups (magnification X 50). The colon length and damage scores of the groups are shown in Figure 6B. The *E.limosum*

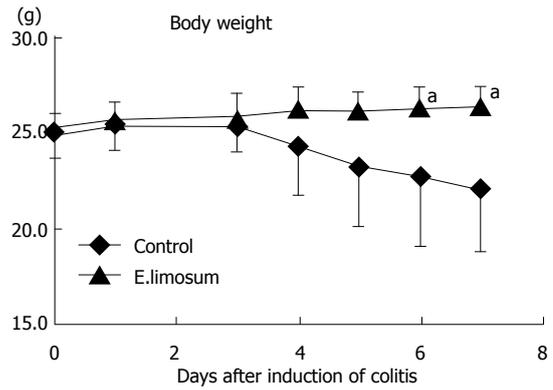


Figure 4 Changes in body weight after initiation of dextran sulfate sodium-induced colitis. control indicates the control diet (cellulose diet) group. *E.limosum* indicates the *Eubacterium limosum* diet group. Data is expressed as means \pm SD ($n=7$). ^a $P<0.05$ vs control.

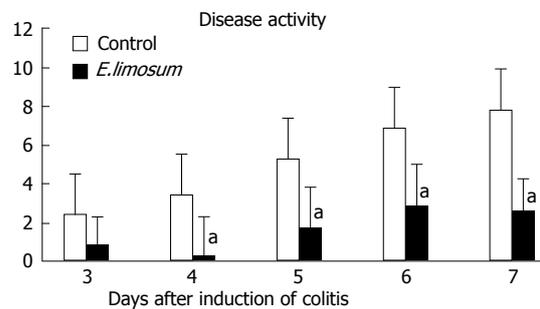


Figure 5 Disease activity index after initiation of dextran sulfate sodium-induced colitis. Control indicates the control diet (cellulose diet) group. *E.limosum* indicates the *Eubacterium limosum* diet group. Data is expressed as means \pm SD ($n=7$). ^a $P<0.05$ vs control. Disease activity index was determined by scoring changes in body weight, hemoccult positivity, and stool consistency.

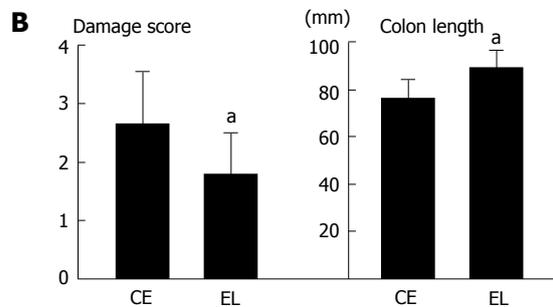
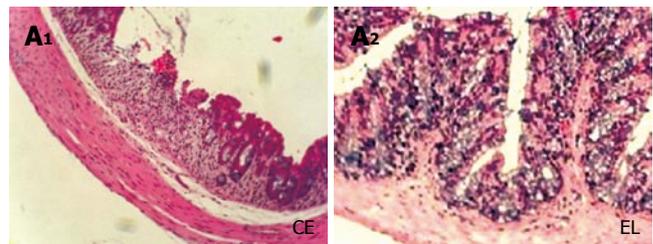


Figure 6 Light histological section of colonic mucosa (A) and changes in colon length and colonic mucosal damage score (B) of dextran sulfate sodium-induced colitis control and *E.limosum* diet groups. (magnification X 50). Control indicates the control diet (cellulose diet) group. *E.limosum* indicates the *Eubacterium limosum* diet group. Data is expressed as means \pm SD ($n=7$). ^a $P<0.05$ vs control.

diet significantly attenuated the mucosal damage and shortening of colon length compared to those in the control group.

DISCUSSION

IBD is considered to be a dysfunctional response of the mucosal immune system to luminal antigens and commensal microbiota in genetically susceptible individuals. Therefore, it is a rationally therapeutic strategy to eliminate the pathogenic bacterial component.^[22,23] It has already been reported that manipulation of microflora by antibiotics, probiotics, and prebiotics was effective in IBD treatment. Antibiotic therapy is recognized to have an essential role in IBD. For example, metronidazole decreases perianal complications in Crohn's disease patients and ciprofloxacin reduces the presence of *Clostridium difficile*, which is an aggravating bacteria in IBD, and improves clinical symptoms.^[24] With regards to the probiotic therapy, it is also considered a rational therapeutic option in IBD. Recent studies have shown that restoring the microbial balance using probiotics might contribute to the normalization of microflora and the attenuation of symptoms in IBD.^[4] Prebiotic administration can also modulate the microflora by increasing the populations of beneficial bacteria and thereby quantitatively changing the composition of the microflora.^[25] These alterations may act beneficially, in part by causing a luminal induction of short-chain fatty acids (SCFAs), which are the major probiotic metabolites, are important nutrients for the intestine, and induce an acidic environment. Especially, butyrate plays a trophic role as a first nutrient for colonocytes.^[12,26] It has also been reported to act as an anti-inflammatory agent by inactivating transcriptional factor (NF- κ B or STAT3).^[12]

GBF stimulated growth of Bifidobacterium species and *E. limosum* and production of its metabolites butyrate and other short-chain fatty acids. Four-week administration of 20 to 30 g of GBF to patients with mild to moderate UC decreased clinical and endoscopic evidence of inflammation in a trial. This treatment increased fecal density of Bifidobacterium species and *E. limosum*.^[8] A longer term (24 wk) open-label study reached similar conclusions.^[13,27] In our previous study, the metabolites of *E. limosum* obtained as the supernatant of the medium after inoculation increased the cell growth rate as determined by SUDH activity and attenuated the production of IL-6 by TNF- α stimulation in T84.^[21] However, the detailed role of *E. limosum* metabolites on mucosa has not been determined.

In this study, the role of the main composition of metabolites, a mixture of organic acids, on mucosa was examined. Except for succinate, the organic acids we used significantly increased the growth activity of T84. Interestingly, the cocktail containing acids at low levels, which had no stimulatory effects, significantly increased the growth rate compared to that in the control. The epithelium may require the coexistence of multiple organic acids to stimulate its growth. The cocktail significantly attenuated IL-6 production as well as the production of all short-chain fatty acids. Compared with a single acid, multiple, coexisting organic acids may increase the barrier function and anti-inflammatory effects on inflammatory colonic mucosa at a relatively low level. In the future, we need to evaluate the detailed mechanism of organic acids

on mucosa, while considering the influence of the other, soluble unknown factors in metabolites.

In this study, a powder of *E. limosum* was administered to experimental colitis model, and an *E. limosum* diet significantly prevented body-weight loss and disease activity compared with the control group. Also, the *E. limosum* diet dramatically increased the levels of 3 kinds of short chain fatty acids and 2 organic acids. As described in the method section, we could not evaluate the microbial data in cecal content, because its volume was very low. In addition, it is still not clear that the net viability in gastrointestinal tract and resistance to gastric or bile acids was derived from its obligate anaerobic character. It is speculated that the number of dietary *E. limosum* changed (decreased) day by day, in the diet since this bacterium is an obligate anaerobe and may have been adversely affected by ambient oxygen exposure during dietary administration. Care was exercised in the preparation of sufficient *E. limosum* due to its slow growth rate and culture yields. Additional microbiological data is required to determine precise growth requirements of this microbiota.

However, the present data may suggest that *E. limosum* might be able to reproduce in the gastrointestinal tract, although the obligate anaerobe *E. limosum* was treated under aerobic conditions for use in as an animal diet for a relatively long time. The observed increase of cecal organic acid may strongly support this conjecture.

Microbiota itself contains many kinds of chemical component (peptide-glycan, enzyme, nucleic acid, protein, lipids, and so on). It is very difficult to identify the active component responsible for the anti-inflammatory effect in *E. limosum*. At first, we pointed out its metabolites, especially butyrate in this study. As shown in a previous study, butyrate demonstrated unique characteristics in colitic rats fed GBF.^[9]

The monolayer of intestinal epithelial cells (IEC) plays a role in sensing the external gastrointestinal environment. Disruption or breakdown in the signaling function of IEC is important in the development of IBD.^[28] It was reported that IEC recognized pathogen or bacteria is based on the conserved signature molecules in microbes (microbe or pathogen-associated molecular patterns; PAMPs).^[28,29] PAMPs are found in lipopolysaccharides (LPS) of gram-negative bacteria, and lipoteichoic acids (LTA) of gram-positive bacteria. One of pattern recognition receptor families mediated the response of immune cells to PAMP is TLR family. TLR4 and TLR2, which recognize LPS, peptideglycan and LTA, respectively were well studied. In this study, we investigated the role of the major metabolites butyrate by *E. limosum* on the mucosal innate immune response *via* TLR4. It is very difficult to isolate the cell wall components of *E. limosum* and to evaluate the respective components, since these components might be modified or hydrolyzed by other microflora in gastrointestinal tract, so that, first of all, we applied the metabolites butyrate.

Remarkably butyrate decreased the expression of TLR4 mRNA level with decrease of IL-6 production in T84 cell line. Since a detailed mechanism is still unclear, we needed to evaluate of the role of butyrate in different concentrations or cell lines. However, it was considered

that butyrate had an innate immune modulatory effect.

With regards to the cell wall fraction of *E. limosum*, we do not have experimental data, and a detailed study will be needed, however, an unidentified peptidoglycan or a CpG-DNA fraction derived from *E. limosum* may contribute to the colonic mucosal immune response via TLR 2 or 9.^[30] In our previous preliminary study, GBF peculiarly decreased the number of CD4+ lymphocytes in the colonic intrarepithelial layer and lamina propria in RAG2-/- CD45RB high-transfer colitis mice, together with an increase of cecal butyrate content.^[31] It might also be considered that an increase of butyrate production is evidence of the presence of modulating microflora in this chronic colitis model. Considering the above results, the effects of GBF on colitis could be partly caused by a change in the mucosal immune system via modulation of microflora and its metabolic organic acids, especially butyrate production.

In this paper, we described the effects of the special bacteria (*E. limosum*) and its metabolites on colitis and colonic epithelium. In research regarding the pathogenesis of IBD and medical therapeutic approaches to IBD, we may have to consider the role of microflora and the interaction between epithelium and bacteria in the intestinal tract in more detail. Although these strategies hold great promise and appear to be useful in certain settings, more studies for example, the relationship between mucosa and microflora and its metabolites will be needed. This therapy may provide a new nutraceutical approach to IBD treatment.

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BASIC RESEARCH

Chronic low vitamin intake potentiates cisplatin-induced intestinal epithelial cell apoptosis in WNIN rats

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Abstract

AIM: To investigate if cisplatin alters vitamin status and if VR modulates cisplatin induced intestinal apoptosis and oxidative stress in Wistar/NIN (WNIN) male rats.

METHODS: Weanling, WNIN male rats (n = 12 per group) received *ad libitum* for 17 wk: control diet (20% protein) or the same with 50% vitamin restriction. They were then sub-divided into two groups of six rats each and administered cisplatin (2.61 mg/kg bodyweight) once a week for three wk or PBS (vehicle control). Intestinal epithelial cell (IEC) apoptosis was monitored by morphometry, Annexin-V binding, M30 cytodeath assay and DNA fragmentation. Structural and functional integrity of the villus were assessed by villus height / crypt depth ratio and activities of alkaline phosphatase, lys, ala-dipeptidyl amino-peptidase, respectively. To assess the probable mechanism(s) of altered apoptosis, oxidative stress parameters, caspase-3 activity, and expression of Bcl-2 and Bax were determined.

RESULTS: Cisplatin *per se* decreased plasma vitamin levels and they were the lowest in VR animals treated with cisplatin. As expected VR increased only villus apoptosis, whereas cisplatin increased stem cell apoptosis in the crypt. However, cisplatin treatment of VR rats increased apoptosis both in villus and crypt regions and was associated with higher levels of TBARS, protein carbonyls and caspase-3 activity, but lower GSH concentrations. VR induced decrease in Bcl-2 expression

was further lowered by cisplatin. Bax expression, unaffected by VR was increased on cisplatin treatment. Mucosal functional integrity was severely compromised in cisplatin treated VR-rats.

CONCLUSION: Low intake of vitamins increases the sensitivity of rats to cisplatin and promotes intestinal epithelial cell apoptosis.

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Key words: Apoptosis; Cisplatin; Intestinal epithelium; Mucosal integrity; Oxidative stress; Vitamins.

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INTRODUCTION

Although vitamin deficiency is encountered infrequently in developed countries, inadequate intake of several vitamins is associated with chronic diseases like cancer, coronary heart disease and osteoporosis^[1]. About 35% of all cancers are associated with various nutritional deficiencies or excesses and a poor nutritional status of various water and fat-soluble vitamins has been demonstrated in populations more prone to cancers, without symptoms of clinical vitamin deficiencies^[2]. In addition, the prospect that high intake of certain vitamins may confer protection against cancer and its side effects during chemo/radiotherapy has drawn substantial attention during the last decade^[3]. In this context, we have recently shown that low intake of vitamins (50% reduction) *per se* can increase the intestinal epithelial cell apoptosis through increased oxidative stress and result in lowered functional integrity of the intestinal mucosa^[4,5].

The cytotoxic actions of chemotherapeutic agents are not tumor specific, and injury to normal but rapidly proliferating cells in the bone marrow and intestinal crypt often complicates the treatment of patients with neoplastic disease^[6, 7]. Systemic chemotherapy exerts cytoablative actions via several different mechanisms, ultimately leading to cell cycle arrest and/or apoptosis and produces changes

in the structure of the intestinal mucosa^[8-10] that are associated with increased permeability of the intestine^[11].

Cisplatin (cis-diamminedichloroplatinum, II) is one of the most frequently used anticancer drugs. The therapeutic efficacy of cisplatin derives from its ability to form complexes with DNA^[12], which exert their cytotoxicity by directly inhibiting DNA and RNA synthesis and inducing apoptosis^[13,14]. In addition, cisplatin has been shown to induce production of reactive oxygen species (ROS) that are reported to be important mediators of stress response in many cell types^[15-17]. ROS accumulation and reduced glutathione (GSH) levels are critical to the bioactivity of cisplatin^[18-20]. Importantly, inhibitors of ROS can block cisplatin-induced apoptosis indicating that pathways involved in and/or activated by oxidative stress are critical to cisplatin bioactivity^[17]. In fact, increased intracellular GSH concentrations are found in cells resistant to cisplatin^[18], and cisplatin-induced toxicity can be blunted by systemic administration of antioxidants like N-acetyl cysteine^[20]. It has been demonstrated earlier that mitochondrial damage is an early event in the pathogenesis of gastric toxicity due to cisplatin^[21]. However, cisplatin-induced apoptosis also involves events that are not ROS-dependent^[22].

Whether vitamin status can modulate apoptosis in normal proliferating cells is particularly important for cancer chemotherapy as it has immediate clinical ramifications. Therefore, we have investigated the effect of sub-optimal intake of vitamins mimicking a general fall of vitamin status seen in cancer patients on the intestinal toxicity of cisplatin using rat as an animal model.

MATERIALS AND METHODS

Chemicals and reagents

Cisplatin was obtained from Dabur Pharmaceuticals, India. Annexin V-Biotin, M30 CytoDEATH and Streptavidin peroxidase were procured from Roche Diagnostics, Mannheim, Germany. Primary antibodies for Bcl-2 and Bax were from Oncogene Research products, San Diego, CA, USA and the substrate for caspase-3 (Ac-DEVD-pNA) was from Calbiochem, San Diego, CA, USA. Biotinylated secondary antibodies, RNase, proteinase K, Nonidet NP-40, agarose, lys, ala- 7-amido-4-methyl coumarin and the vitamins used in the diets were from Sigma chemical company, St Louis, MO, USA. All other chemicals were of the highest analytical grade and procured from local sources.

Animals and study design

The animal studies were approved by the Ethical committee for animal experimentation at the National Institute of Nutrition, Hyderabad, India, which ensures that the guidelines set by the government of India in this regard are strictly implemented. A total of 24 Wistar/NIN (WNIN) weanling male rats were obtained from the National Center for Laboratory of Animal Sciences at National Institute of Nutrition, Hyderabad. They were divided randomly into two groups of 12 animals each and were housed individually in polypropylene cages in a room maintained at $24 \pm 2^\circ\text{C}$, 50-60% relative humidity, with

a 12 h light-dark cycle. They were fed for 17 wk, a casein based (20% protein) control diet or the same with 50% restriction of vitamin mixture^[23]. After 17 wk, control and vitamin restricted (VR) rats were further divided into two groups of six rats each and administered cisplatin (CDDP - dissolved in PBS, at a dose of 2.61 mg/kg bodyweight) once in a week for three wk or PBS (vehicle control). The rats had free access to food and water and their food intake (daily) and body weights (weekly) were recorded periodically during the course of the experiment.

At the end of the feeding and treatment regimen, venous blood was collected from all rats after a 17 h fasting, through orbital sinus vein puncture into heparin containing vials. Plasma was separated immediately and stored at -20°C for analysis of riboflavin, folic acid and vitamin E. Three rats from each group were euthanized in a CO₂ chamber and sacrificed each day. A 20-cm segment of the jejunum, beginning 12-cm distal to the ligament of Treitz, was immediately excised via a midline abdominal incision and freed from mesentery and fat. It was then processed for evaluating the changes in apoptotic rates.

Processing of jejunum

The 20-cm segment of jejunum was gently washed with ice-cold phosphate buffered saline (PBS), divided randomly into three segments of 5, 5 and 10 cm each and processed as reported earlier^[14]. They were used respectively for light microscopic observations, isolation of epithelial cells and extraction of DNA and determination of enzyme activities, parameters of oxidative stress and antioxidant status in addition to the expression of pro and anti apoptotic proteins, as described earlier^[14,15].

Detection of apoptosis

Samples of rat jejunum fixed in buffered formalin for 24 h were dehydrated, embedded in paraffin and the number of apoptotic cells were scored in 4- μm thick serial sections under a light microscope, after: (i) staining with hematoxylin and eosin (ii) M30 staining and (iii) by Annexin V binding, as described by us earlier^[14,15]. Also, the DNA isolated from the jejunal epithelial cells was resolved on an agarose gel and the DNA fragments visualized under a UV trans-illuminator after ethidium bromide staining (BioRad).

Oxidative stress, antioxidant status and expression of proteins modulating apoptosis

The frozen samples of the mucosal scrapings were processed and the activities of caspase-3, Cu, Zn-SOD, glutathione peroxidase, catalase were determined as described by us earlier^[14,15]. Tissue oxidative stress was quantified by the estimation of TBARS and protein carbonyls and the expression of apoptotic modulatory proteins Bcl-2 and Bax was assessed by immunoprecipitation and western blotting^[14,15].

Structural and functional integrity of villus

Structural integrity of the villus was assessed from the ratio of villus height: crypt depths, which were measured under a light microscope (100 \times magnification) using a calibrated ocular micrometer. The values of the villus height and

Table 1 Plasma levels of fat and water-soluble vitamins in control and vitamin restricted rats treated with cisplatin

| Group | Folate ($\mu\text{g}/\text{dL}$) | Riboflavin ($\mu\text{g}/\text{dL}$) | α -Tocopherol ($\mu\text{g}/\text{dL}$) |
|----------|------------------------------------|--|--|
| CON | 4.85 ^a ±0.10 | 4.74 ^a ±0.06 | 74.1 ^a ±2.59 |
| VR | 2.08 ^b ±0.50 | 2.42 ^b ±0.12 | 46.4 ^b ±2.74 |
| CON+CDDP | 4.23 ^c ±0.11 | 4.05 ^c ±0.07 | 63.2 ^c ±2.93 |
| VR+CDDP | 1.28 ^d ±0.11 | 1.85 ^d ±0.03 | 33.1 ^d ±2.61 |

Values are mean \pm SE of 6 observations; Means in a column with different superscripts are significantly different from each other ($P \leq 0.05$), by one-way ANOVA, followed by post-hoc least significant difference (LSD) test. CON - control rats; VR - vitamin restricted rats; CON+CDDP - control rats treated with cisplatin and VR+CDDP - vitamin restricted rats treated with cisplatin.

crypt depth in each animal were obtained from the mean of measurements made in seven different fields of the tissue section as described earlier [14]. Activities of alkaline phosphatase and lys, ala dipeptidyl amino peptidase were measured 12000 g supernatant, as markers of functional integrity of the mucosal membrane [14, 15].

Plasma vitamin status

Plasma vitamin E and riboflavin levels were quantified on an RP-HPLC (Agilent Tech Inc., CA, USA) [14], whereas folic acid was determined by a solid phase competitive binding assay as described earlier [15].

Statistical analysis

All the results are expressed as mean \pm SE. Data was analyzed statistically by one way analysis of variance (ANOVA) followed by Post Hoc multiple comparison tests of significance using the SPSS package (Version 10.0, Chicago, USA). Since no heterogeneity of variance was observed with any of the parameters tested, differences among the groups were tested by the parametric, least significant difference (LSD) test. The differences were considered significant only if $P < 0.05$.

RESULTS

Body weight

Feeding vitamin restricted diet for 17 wk showed no significant changes in body weight of rats compared to controls. However, cisplatin administration during the next 3 wk resulted in loss of body weight to an extent of 10 % in control rats and 18 % in VR rats, due to reduced food intake (data not given).

Plasma vitamin status

Plasma concentration of folate decreased by 13 % in control rats treated with cisplatin compared to those treated with saline. On the other hand they decreased by 38% in VR rats treated with cisplatin compared to saline treatment. Plasma riboflavin concentration decreased by 14% in cisplatin treated compared to saline treated control rats, where as this decrease was 24% in cisplatin treated compared to saline treated VR rats. Plasma vitamin E level fell by 15% in control rats treated with cisplatin compared to those treated with saline, where as the decrease was

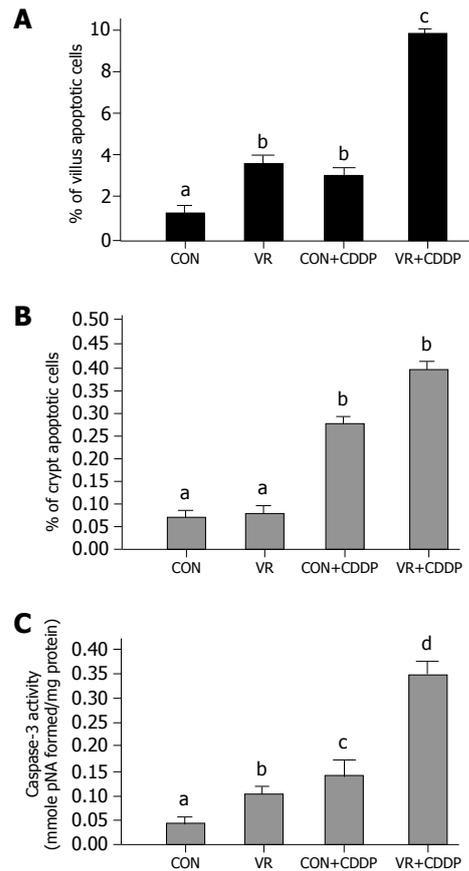


Figure 1 Apoptotic index and caspase-3 activity of the intestinal mucosa of control and vitamin restricted rats treated with cisplatin or saline (vehicle). Vertical bars are means with standard error, of IEC apoptotic indices in villus (Panel A) and crypt (Panel B) regions of control, vitamin restricted rats treated with saline and cisplatin ($n = 6$ per group). Intestinal sections were stained with hematoxylin and eosin and studied by morphometry under a light microscope. Caspase-3 activity (Panel C) was measured in 12000 g supernatant using a colorimetric substrate.

29 % in cisplatin treated compared to saline treated VR rats (Table 1).

Jejunal mucosal apoptosis

Vitamin restriction *per se* increased the jejunal villus apoptosis by 2 - fold, but not the crypt apoptosis. Cisplatin increased jejunal villus apoptosis by 1.7 and 2.4 fold respectively in control and VR rats (Figure 1A). However, crypt apoptosis in jejunum increased by 4 and 5 fold in control and VR rats respectively by cisplatin treatment (Figure 1B). Vitamin restriction *per se* increased Caspase - 3 activity by 2.4-fold. Cisplatin treatment increased caspase-3 activity by 3.5-fold in control and VR rats, compared to their saline treated controls (Figure 1C). There were 14% Annexin V positive cells in VR rats. Cisplatin treatment resulted in 13 and 25 % Annexin V positive cells in control and VR rats respectively (data not given). On the other hand M 30 positive cells were 25% in VR rats. Cisplatin treatment resulted in 30% of cells positive for M 30 in control rats whereas M 30 positive cells were 70% in VR rats (Figure 2). VR rats showed a clear inter-nucleosomal DNA fragmentation pattern characteristic of apoptosis. Interestingly DNA fragmentation increased further with cisplatin treatment.

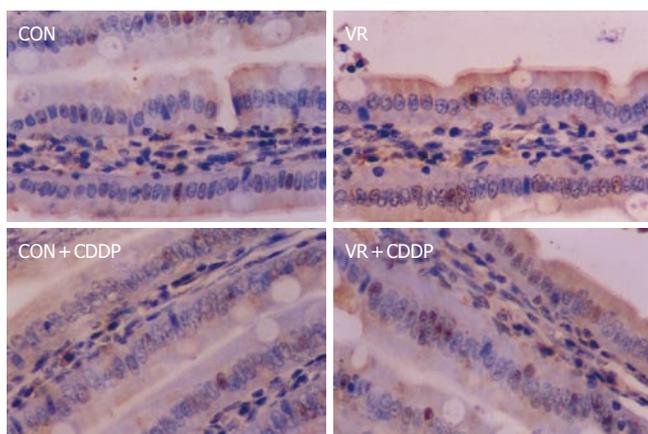


Figure 2 Photomicrographs of apoptotic cells showing positivity for M30 antibody. Representative photomicrographs of intestinal sections immuno-histochemically stained with M 30 antibody. De-paraffinized, intestinal sections were hydrated and probed with M 30 antibody and the positive cells detected by enzymatic color reaction. Number of the brown colored positive cells were counted in a total of 1000 cells and expressed as the percentage.

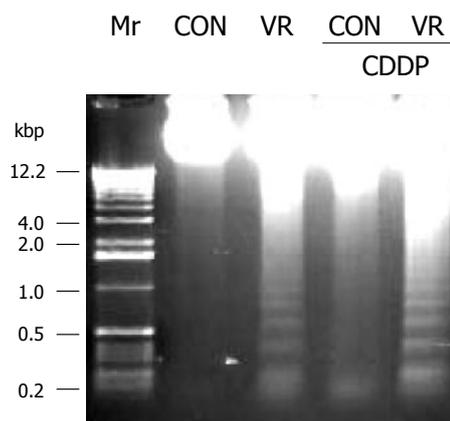


Figure 3 DNA fragmentation pattern in intestinal epithelial cells of control and vitamin restricted treated with saline or cisplatin. Epithelial cells were separated from a portion of rat jejunum, DNA extracted and resolved on a 1.5% agarose gel and stained with ethidium bromide.

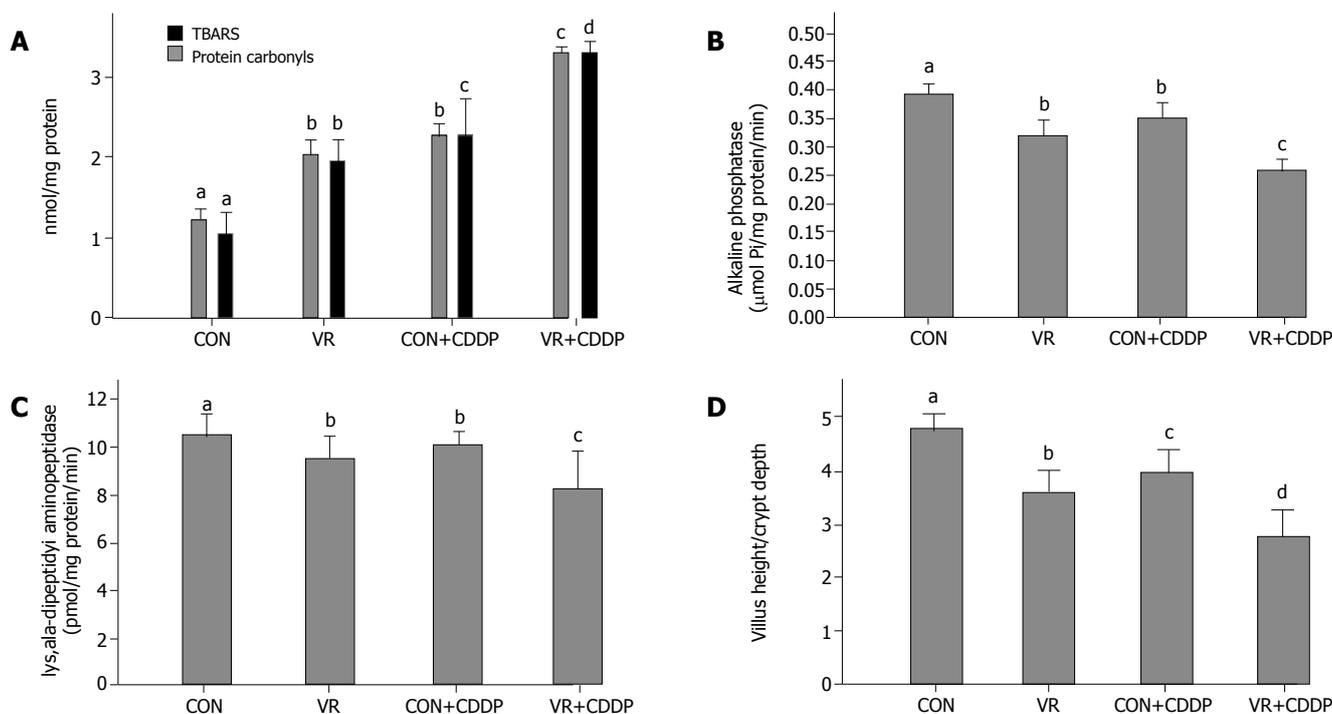


Figure 4 Effect of vitamin restriction and cisplatin on oxidative stress, structural and functional integrity of the intestinal mucosa. Levels of lipid peroxides (TBARS) and protein carbonyls are shown in panel A. Lowered activities of alkaline phosphatase (Panel B), lys, ala-dipeptidyl aminopeptidase (Panel C), are indicative of compromised functional integrity. Panel D indicates the ratio of villus height: crypt depth in control and vitamin restricted rats treated with saline and cisplatin, indicating structural integrity of the mucosa.

However, control rats treated with cisplatin showed relatively less fragmentation of DNA compared to VR rats (Figure 3).

Tissue oxidative stress

Levels of TBARS and protein carbonyls in intestinal epithelial cells increased significantly with vitamin restriction compared to control rats. Cisplatin administration increased the levels of TBARS and protein carbonyls both in control and VR rats, but to a lesser extent in control than VR rats (Figure 4A).

Structural and functional integrity of the jejunal villi

Activities of alkaline phosphatase and lys, ala - dipeptidyl amino-peptidase decreased significantly in the intestinal epithelial cells of rats fed vitamin restricted diet. Cisplatin treatment decreased their activities not only in control rats but also decreased it further in VR rats, compared to their saline treated controls (Figure 4B, C). Ratio of Villus height to Crypt depth, indicative of structural integrity of the intestinal mucosa, significantly decreased with vitamin restricted diet and showed a further decline with cisplatin treatment (Figure 4D).

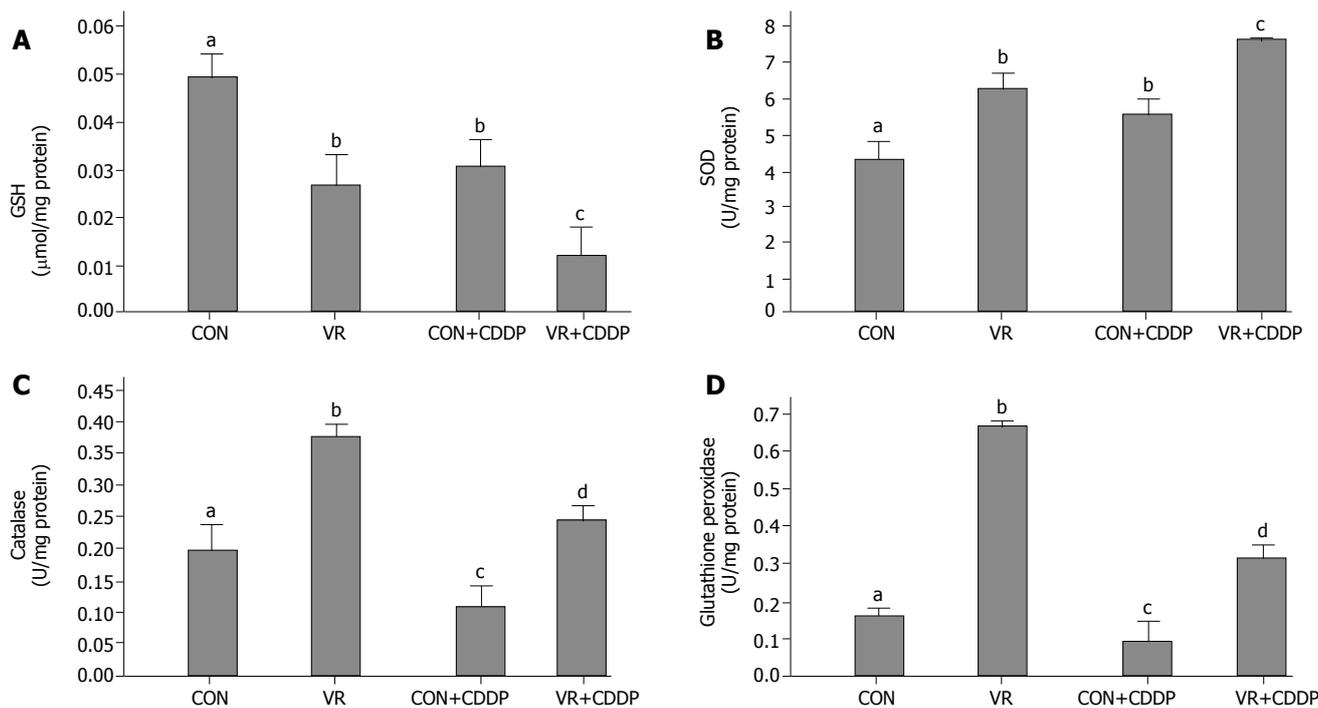


Figure 5 Intestinal glutathione levels and antioxidant enzyme activities in control and vitamin restricted rats treated with saline or cisplatin. Glutathione (GSH) levels are shown in panel A, whereas activities of SOD, catalase and glutathione peroxidase (GPX) are shown in panels B to D, respectively.

Tissue antioxidant status

Vitamin restriction significantly decreased the intestinal epithelial cell GSH concentration compared to control rats. Cisplatin treatment *per se* lowered GSH levels in control rats compared to saline treated ones. Cisplatin treatment further lowered the GSH levels in VR rats compared to saline treated VR rats (Figure 5A).

Vitamin restriction as well as cisplatin administration increased the SOD activity significantly (Figure 5B). Catalase activity increased significantly in VR rats compared to control rats. Cisplatin administration in control and VR rats significantly decreased the catalase activity compared to saline treated rats (Figure 5C). Glutathione peroxidase activity increased significantly with vitamin restriction. Similar to catalase, cisplatin administration in control and VR rats significantly decreased the glutathione peroxidase activity compared to saline treated rats (Figure 5D).

Expression of pro- and anti-apoptotic proteins in intestinal epithelial cells

Figure 6 shows the levels of Bcl-2 and Bax in control and vitamin restricted rats treated with saline or cisplatin. Cisplatin treatment as also vitamin restriction decreased Bcl-2 levels significantly. The levels decreased further in VR rats on cisplatin treatment. On the other hand Bax levels increased significantly in VR rats compared to controls. Cisplatin treatment elevated the levels of Bax protein not only in control rats but also increased them further in VR rats.

DISCUSSION

Low intake of vitamins increased cisplatin action on epithelial cell apoptosis

Cisplatin is a free radical-producing drug that significantly

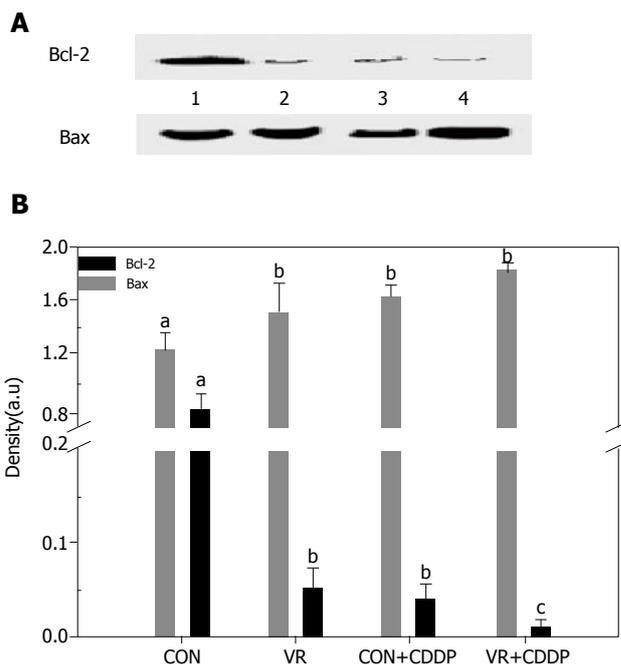


Figure 6 Expression of apoptosis regulatory proteins (Bcl-2 and Bax) in the intestinal epithelial cells Bcl-2 and Bax were immune-precipitated from the 12,000 g supernatant of the intestinal mucosal scrapings using monoclonal Bcl-2 / Bax antibodies. The immuno-precipitates were resolved on 12% SDS-PAGE, transferred electrophoretically to PVDF membrane and the immuno-blot developed using the same antibodies and detected / quantified with HRP conjugated anti - rabbit antibodies. Lane 1 - 4 are: CON, VR, CON+CDDP, VR+CDDP respectively. Upper panel shows Bcl-2 expression and bottom panel shows Bax expression. Panel B: Quantification of the IP / Western blots of Bcl-2 and Bax proteins. Immuno-blot was scanned using a densitometer (BioRad) and densities quantified using the Quantity One software from BioRad. Each bar represents the mean ± SE of three immuno-blot. Bars with different superscripts are significantly different from one another ($P \leq 0.05$) by one-way ANOVA and post-hoc least significant difference test.

decreases the plasma concentrations of antioxidants (24) and induces intestinal epithelial cell apoptosis. Earlier, we showed that chronic low intake of vitamins decreased the plasma vitamin status and significantly increased intestinal villus epithelial cell apoptosis compared to control rats (14). In line with these findings, feeding 50% vitamin restricted diet for 20 wk significantly lowered the plasma levels of both fat and water soluble vitamins, with no significant effect either on food intake or body weight (data not shown). Interestingly, cisplatin administration *per se* decreased the plasma vitamin levels to about 85% of controls and it reduced further, the plasma vitamin levels in VR rats to 30-40% of controls. These results indicate that cisplatin treatment *per se* impairs the vitamin status, but also worsens the vitamin status in VR rats further.

Therefore, to understand if reduced vitamin status has any role in cisplatin induced intestinal toxicity, we assessed IEC apoptosis in control and VR rats treated with cisplatin. Unlike vitamin restriction, which increased the villus apoptosis alone, cisplatin increased apoptosis both in villus and crypt regions by 2.3 and 4.0- fold respectively (over saline treated controls) as assessed by morphometry. On the other hand, cisplatin treatment of VR rats increased villus and crypt apoptosis by 2.7 and 5.0- fold (over saline treated VR rats) respectively (Figure 1A and B). In line with earlier reports that chemotherapy causes intestinal damage with apoptosis of intestinal crypts, which precedes hypo proliferation resulting in crypt hypoplasia^[25], the stem cell region (crypt) showed greater sensitivity to cisplatin both in control and VR rats. Although VR *per se* had no effect on crypt apoptosis, it appeared to sensitize the crypt region to cisplatin induced apoptosis and in the villus region, VR appeared to synergistically increase cisplatin induced apoptosis. These results show for the first time to the best of our knowledge, different effects of VR on cisplatin induced apoptosis in proliferating and differentiated cells of the intestinal mucosa. Higher rates of apoptosis as assessed by morphometry correlated well with increased staining of IECs with Annexin V (data not shown) and M 30 antibodies (Figure 2). Indeed, 70% of the IECs were M 30 positive in VR rats treated with cisplatin while only 30% were positive in control rats treated with cisplatin.

Caspase 3 is an effector caspase activated by many apoptotic stimuli. To confirm that the increased IEC death in cisplatin treated VR rats is truly apoptotic and not necrotic, we assessed whether cisplatin and VR induced increase in IEC apoptosis correlated with an increase in effector caspase activation. Indeed caspase - 3 in VR rats was activated about 2.4 fold over controls whereas it increased about 3.0 fold both in control and VR rats treated with cisplatin (Figure 1C). Further, the increased Caspase 3 activity is in line with the synergistic effect of VR on cisplatin induced IEC apoptosis. That cisplatin treated VR rats showed 8-fold increase in caspase-3 activity compared to saline treated controls (Figure 1C) corroborates the increased IEC apoptosis in cisplatin treated VR rats and confirms that low intake of vitamins increases the sensitivity of intestinal epithelium to cisplatin induced apoptosis.

Modulation of the sensitivity of IECs to cisplatin-induced apoptosis by VR was further assessed by DNA

fragmentation in these cells. Despite a constitutive low rate of apoptosis in IECs of control rats, no DNA fragmentation was detectable, where as low amounts of fragmented DNA were observed in VR rats (Figure 3). Cisplatin treatment *per se* significantly increased DNA fragmentation, while priming with low vitamin intake further increased the abundance of DNA fragments.

Increased apoptosis is associated with increased oxidative damage of protein and lipids in intestinal epithelial cells

Since VR increases IEC apoptosis by increasing oxidative stress, we determined whether cisplatin also affects the IEC oxidative stress. The significantly higher levels of TBARS and protein carbonyls, the oxidative products of lipids and proteins, in the IECs of cisplatin treated control rats indicates increased oxidative stress in them (Figure 4A). That their levels increased further and were the highest in cisplatin treated VR rats indicates that VR modulated cisplatin induced oxidative stress. The data suggests that cisplatin treatment substantially increased the ROS formation in the intestinal mucosa.

Vitamin restriction and cisplatin administration compromised the structural and functional integrity of villi

In view of our earlier finding that VR induced increase in IEC apoptosis affected the structural and functional integrity of the intestinal mucosa^[14, 15], we assessed next, whether the increased IEC apoptosis induced by cisplatin in control and VR rats has similar effects. Cisplatin administration significantly decreased the ratio of villus height / crypt depth (Figure 4D) as well as mucosal marker enzyme activities (Figures 4B & C) in IECs of control rats and these indices were decreased further in cisplatin treated VR rats, indicating altered structural and functional integrity of mucosa and the synergism between cisplatin treatment and VR in this regard. These results indicate that cisplatin *per se* increases oxidative stress in general and lipid peroxidation in particular, which damage the membranes facilitating the leakage of solutes and VR appears to enhance this effect further. This appears to be responsible at least in part, for the lowered vitamin status in cisplatin treated rats.

Lowered antioxidant status and an imbalance in antioxidant enzyme activities with vitamin restriction increased mucosal sensitivity to cisplatin action

To gain insight into the oxidant-antioxidant balance in rats treated with cisplatin and the effect of VR on it, we monitored the GSH levels in addition to determining the antioxidant enzyme activities. Elevated intracellular levels of GSH, the cellular antioxidant involved in free radical scavenging activity, is associated with resistance to chemotherapeutic agents^[26]. VR *per se* decreased the GSH levels in the IECs and cisplatin further decreased them markedly. (Figure 5A). Endogenous antioxidant systems are of singular importance in limiting oxidative cellular damage. To assess the increased sensitivity if any, of intestinal epithelium to cisplatin in VR rats was also associated with altered activities of anti-oxidant enzymes, we monitored the activities of antioxidant enzymes. Vitamin restriction *per se* increased the activities of all the

three enzymes. While cisplatin treatment increased SOD and decreased catalase and GPX activities in control rats, it increased SOD further, and significantly decreased catalase and GPX activities in VR rats. These changes in the antioxidant enzymes observed in this study are in general agreement with the following literature reports: Up regulation / over expression of SOD and catalase are known to decrease the toxicity of cisplatin^[27], whereas down regulation of GPX is associated with mitochondrial dysfunction in the kidney epithelial cells^[28]. It appears that the possible increase in the accumulation of H₂O₂ or R₂O₂ as a consequence of the imbalance in the antioxidant enzyme activities could enhance the intrinsic sensitivity of the intestinal mucosa to cisplatin-induced apoptosis.

Vitamin restriction and cisplatin administration lowered Bcl-2 and increased Bax expression

To determine, if the increased IEC apoptosis seen in vitamin restriction and cisplatin treatment is associated with altered expression of the pro and anti-apoptotic proteins, the levels of Bax and Bcl-2 were measured by IP - Western blotting. Whereas Bcl-2 expression significantly decreased in VR rats (than controls), it was barely detectable in cisplatin treated control rats (Figure 6A). On the other hand, Bax expression was not altered in VR rats, but cisplatin increased its expression both in control and VR rats, albeit the increase was not significant (Figure 6B). The increased Bcl-2 expression is shown to stabilize mitochondria, prevent cytochrome c release and thereby reduce the cytotoxic potential of most anticancer drugs^[29]. These results thus seem to suggest that up-regulation of Bax and the down-regulation of Bcl-2 were involved in cisplatin induced IEC apoptosis and that VR may not affect the action of cisplatin in this regard.

Anticancer drugs are known to operate principally by "forcing" the actively proliferating tumor cells into apoptosis^[30]. However, these drugs also affect other proliferating cells like stem cells in the crypt of the intestinal mucosa and bone marrow either due to altered cellular redox status and / or deregulation of pro and anti-apoptotic protein expression. Since our studies indicate such effects to be more pronounced in vitamin restriction and that cisplatin treatment impairs vitamin status, it appears possible that intestinal toxicity of anticancer drugs can at least partly be due to a fall in vitamin status caused by cancer chemotherapy. Further, as VR potentiates the apoptotic response of IECs to cisplatin, vitamin deficiencies common among cancer patients may alter their intestinal tissue homeostasis and when they are exposed to a stress like chemical or radiation insult, result in frank tissue injury and loss of functional integrity. Our results provide qualitative and quantitative evidence, which support the hypothesis that vitamin restriction markedly amplifies the toxicity of cisplatin in the intestine. Therefore the need to fix the dose of anticancer drug based on the subjects' vitamin status appears imperative to avoid the toxicity of the drug to the normal IECs. Further, it appears pertinent to study whether vitamin supplementation either singly or as a mixture can reverse such changes associated with cisplatin administration.

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BASIC RESEARCH

Reduced antioxidant level and increased oxidative damage in intact liver lobes during ischaemia-reperfusion

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antioxidant defence system. Al, Cu, Mn, Zn, and S were lower in the hyperemia group than in the sham operated group when the levels of Ca, Fe, Mg, Se and P ions were higher during hyperemia.

CONCLUSION: Oxidative stress is one of the main factors for the injury of intact liver lobes during ischaemia-reperfusion.

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Key words: Free radicals; Liver; Oxidative stress; Metal elements; Redox-balance

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Abstract

AIM: To determine whether increased blood flow of the liver can cause oxidative stress and hepatocyte damage, and to elaborate methods suitable for measuring the antioxidant defence during hepatic surgery on rat model.

METHODS: In nembutal narcosis, the left lateral and the medial lobes of the liver were clipped for 45 min to make the total blood supply flow through the other lobes. Total antioxidant status, glutathione peroxidase and superoxide dismutase activity, as well as the concentrations of diene conjugates and free sulphhydryl groups, H-donating ability and reducing power of the liver samples were determined. Chemiluminescent intensity of the liver was also measured. Metal ions (Al, Ca, Cu, Fe, Mg, Mn, Zn) and P and S concentrations of the liver were determined with an inductively coupled plasma optical emission spectrometer and Se content was measured by cathodic stripping voltammetry.

RESULTS: Glutathione peroxidase and superoxide dismutase activities of the liver decreased significantly in the hyperemia group compared to those observed in the sham operated group. The level of total antioxidant status was also significantly lower in the hyperemia group. H-donating ability, reducing power and free sulphhydryl group concentration showed the same tendency. A significant correlation ($P < 0.05$) was found between the changes in non-specific antioxidant activities. This pointed to simultaneous activity of the

INTRODUCTION

Acute hyperemia (hyperperfusion) occurs during operations of the liver when the blood flow of part of the liver is selectively occluded by the surgeon, therefore the non-occluded lobes have to deal with a greater amount of blood. This type of operation is relatively rare, but the mechanism of this kind of damage can be of great interest since very few data on this topic are available in the literature. After liver resection the non-resected liver tissue receives the whole blood volume of the portal vein and the hepatic artery, therefore it may be assumed that hepatic overflow is related to these operations. The same mechanism occurs during liver transplantation from a living donor both in the liver of the donor and in the recipient. Owing to the relatively small size of the liver it is rather difficult to handle hemodynamic changes^[1].

Hemodynamic changes due to the regeneration of the liver may have effect on the above mentioned hepatic hyperperfusion, therefore long-term investigation on this model may be an interesting field of research in the future^[2-4].

Along with the increased blood flow, oxygen tension of the hepatic tissue is also high. High oxygen tension

is connected to the generation of oxygen-related free radicals, such as hydroxyl radical, superoxide-anion or hydrogen peroxide. Oxidative stress causes major disturbances in the redox-homeostasis of the liver cells, therefore it also affects the main cellular functions through signal transduction^[5].

Especially in liver tissue, oxidative stress activates the Kupffer cells and Ito cells first, which are responsible for most of the liver damage^[6]. Great amounts of free radicals are generated by Kupffer cells during their respiratory burst, which is a most important source of reactive oxygen species. Kupffer cells generate other mediators (cytokines, prostaglandins) as well, and these agents increase the damage of the liver tissue^[7].

Oxidative stress causes lipid peroxidation of the cellular membranes, and diene conjugates and malondialdehyde are generated^[8]. The injury of the cellular membranes destroys the homeostasis of hepatocytes, which leads finally to apoptosis or necrosis^[9].

The great amount of free radicals and fast blood flow increase the risk of thrombosis, which can be related to greater liver cell damage^[10]. Platelet aggregation is also affected by superoxide anions and hydroxyl radicals^[11, 12]. The disturbance in the circulation of the liver may cause endothelial dysfunction by changing the ratio of vasoconstrictor and vasodilator agents^[13, 14]. The role of the white blood cells in thrombotic mechanisms related to ischaemia-reperfusion is well known. The same reactions may occur in intact liver lobes^[15, 16].

Oxidative stress and the generation of free radicals are closely related to transition metals and the antioxidant defence system is dependent on metal elements and selenium^[17, 18]. Therefore, determination of the element content in liver is relevant.

The aim of this study was to determine whether increased blood flow of the liver may cause oxidative stress of hepatocytes and to find methods suitable for measuring antioxidant defence during hepatic surgery on rat model.

MATERIALS AND METHODS

Materials

Luminol, microperoxidase, hydrogen peroxide, and 1,1-diphenyl-2-picryl-hydrazyl radical were obtained from SIGMA (St. Louis). TAS (NX2332), SOD (SD 125) and GSHPx (RS 506) kits were bought from Randox (Crumlin, UK). The standard solution for ICP measurements was made from Merck ICP standards, and other chemical reagents were purchased from Reanal Chemical Company (Budapest).

Surgical model

Male Wistar rats (250 g) were purchased from Charles River Hungary and housed in a temperature- and humidity-controlled room under a constant 12-h light/dark cycle. Animals had free access to water and standard rat chow. All experiments were performed with rats fasted for 12 h prior to operations. All studies were performed with the permission of the Animal Health and Food Control Station (770/004/04).

Surgery was performed under deep nembutal narcosis (35 mg/body weight kg). Body temperature was kept between 36.5 °C and 37.5 °C. After laparotomy, circulation occlusion of the left lateral and the medial liver lobes was induced. Reflow was initiated by the removal of microclips, which selectively clamped the branches of the portal vein and hepatic artery.

Experimental design

For evaluation of the hepatic overflow injury, a control group and a sham operated group were compared with two intervention groups respectively.

The rats were divided into control group ($n=6$) in which rats were not operated; sham operated group ($n=6$) in which laparotomy was performed for 45 min without damage to the liver; hyperemia (hyperperfusion) group ($n=6$) in which the peduncles of the right, left medial and lateral lobes were clipped to make the total blood volume of the portal vein and the hepatic artery flow through the lesser lobes of the liver for 45 min and then liver samples were taken as reperfusion group ($n=6$) in which the clips were removed from the peduncles of the ligated lobes to restore the original hemodynamic conditions of the liver after the 45-min-hyperemia period, modelling selectively induced ischaemic resection type of the liver. After 15 min of reperfusion the livers were stored at -20 °C for further examinations. The rats were exsanguinated from the arterial vein after the experiments.

Measurement of enzyme activity and concentration of metal ions

Concentration of the diene-conjugates was measured based on the method of AOAC^[19]. Free SH-groups were determined by the Sedlack method based on the Ellmann reaction^[20]. The H-donating ability of the samples was determined by Blois's method modified by Blázovics *et al.*^[21] in the presence of a 1,1-diphenyl-2-picryl-hydrazyl radical. Absorbance of the methanolic DPPH-dye was detected spectrophotometrically at 517 nm. For characterization of the ability, inhibition (%) was given to the DPPH degradation^[22, 23]. Oyaizu's method was adopted for the determination of the reducing power (RP) of the samples. The change in absorbance was measured, which accompanied Fe^{3+} - Fe^{2+} transformation at 700 nm, and the RP was compared to that of ascorbic acid^[24]. All spectrophotometric measurements were carried out with a Jasco V 550 instrument.

A recently developed chemiluminescence assay adapted to a Berthold Lumat 9501 instrument was applied. The procedure was carried out by the method of Blázovics *et al.*^[21]. The volume of liver homogenate samples (protein content was 10 mg/mL) was 0.06 mL. Chemiluminescent intensity of the samples was expressed in RLU% (relative light unit %) of the standard (basic chemical reaction means: H_2O_2 -luminol-microperoxidase reaction).

The levels of total antioxidant status (TAS), superoxide dismutase (SOD) and glutathione peroxidase (GSHPx) were determined from the supernatants of liver homogenates (10 mg/mL protein content) with Randox kits. The results were expressed in arbitrary units calculated

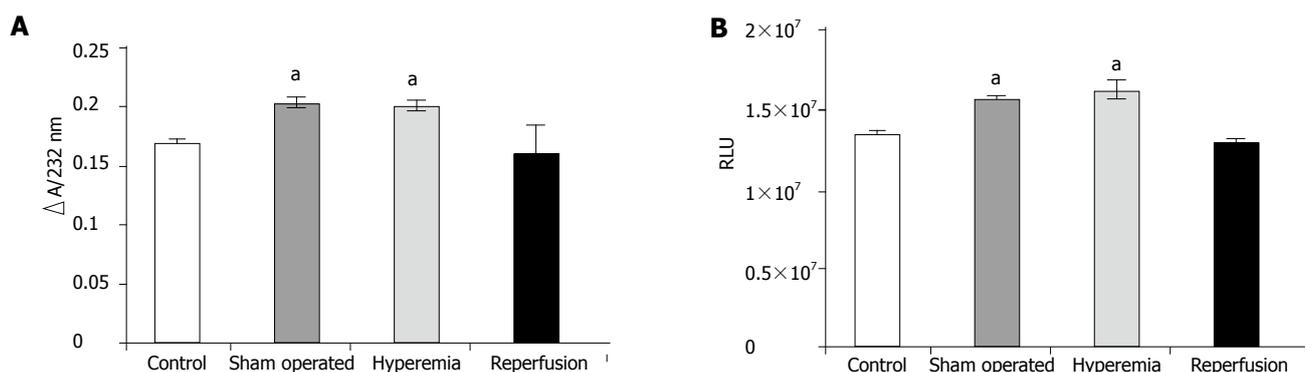


Figure 1 Changes of diene-conjugates (A) and chemiluminescent intensity (B) in different groups. ^a $P < 0.05$ vs control group.

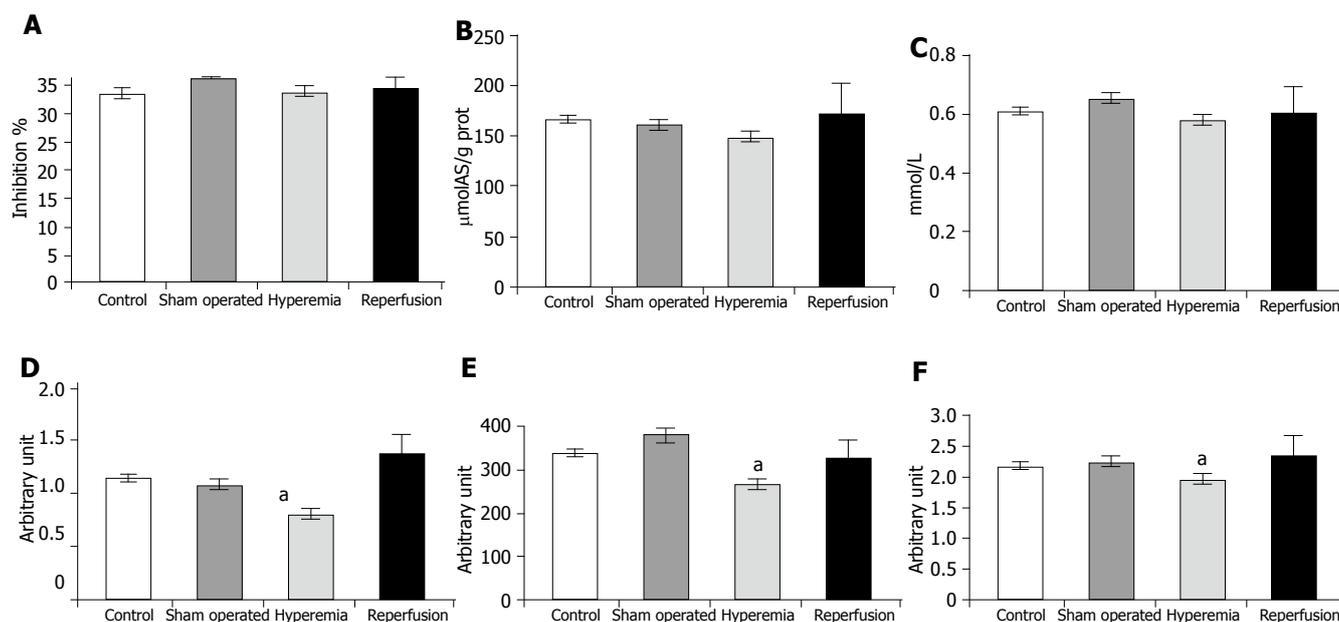


Figure 2 Changes of H-donating ability (A), reducing power (B), concentration of free SH groups (C), SOD activity (D), glutathione-peroxidase activity (E) and TAS level (F) in different groups. ^a $P < 0.05$ vs control group and sham operation group.

from the absorbance detected by spectrophotometry. Protein content was measured by the method of Lowry^[25].

Element concentration in the pooled liver homogenate samples was determined in three parallel measurements with an inductively coupled plasma optical emission spectrometer (ICP-OES). Type of instrument used was Atom Scan 25 (Thermo Jarrell Ash Co.). After digestion of the liver samples (1 mL) with a mixture of nitric acid (5 mL) and hydrogen peroxide (2 mL) diluted to 10 mL with deionised water, the concentration of 9 elements (Al, Ca, Cu, Fe, Mg, Mn, P, S, Zn) was determined three times, three seconds each time. Integration time, blank subtraction and background correction were applied during the measurements^[26].

Se content in the liver was determined by a cathodic stripping voltammetric method with hanging mercury drop electrode (instrument: Trace Lab 50). The digested samples for the element measurement (previous paragraph) was measured in 1 mol/L HCl as supporting electrolyte by preconcentration at -350 mV. Electrolysis time: 100; potential: -300 mV up to -900 mV; step duration; -50 mV/s.

Statistical analysis

One-way ANOVA statistical analysis was performed to evaluate significant levels between the different groups of rats. Pearson's correlation matrix was used to evaluate the correlation between measured parameters. The data were expressed as present mean \pm SD. Five parallel measures were carried out from each of the pooled samples, in this case the data showed mean values when c.v. was below 5.00%. $P < 0.05$ was considered statistically significant.

RESULTS

The amount of generated diene conjugates is shown in Figure 1A. The concentration of diene conjugates increased significantly in the sham operated rats, which was significantly higher in the hyperemia group than that (0.171 ± 0.001) in the control group and (0.201 ± 0.007) in the hyperemia group $\Delta E_{232\text{nm}}$. When the circulation of the liver returned to normal, the level of diene conjugates also tended to return to the normal value.

Table 1 Element concentrations ($\mu\text{g/g}$) in rat liver (mean \pm SD)

| | Control | Sham-operated | Hyperemia | Reperfusion |
|----|------------------|-------------------------------|---------------------------------|------------------------------------|
| Al | 0.501 \pm 0.04 | 0.736 \pm 0.03 [†] | 0.484 \pm 0.03 ^a | 0.643 \pm 0.035 ^{a,c,e} |
| Ca | 22.43 \pm 0.31 | 22.79 \pm 0.04 | 35.05 \pm 0.29 ^{a,e} | 23.72 \pm 0.17 ^{a,c} |
| Cu | 1.628 \pm 0.02 | 1.671 \pm 0.02 | 1.651 \pm 0.01 | 1.595 \pm 0.02 ^{a,c} |
| Fe | 52.04 \pm 1.21 | 46.81 \pm 0.62 ^e | 58.18 \pm 1.08 ^{a,e} | 59.90 \pm 1.03 ^{a,e} |
| Mg | 81.40 \pm 0.29 | 84.60 \pm 1.07 | 89.76 \pm 0.49 ^{a,e} | 81.87 \pm 1.03 ^{a,c} |
| Mn | 1.260 \pm 0.87 | 1.314 \pm 0.02 | 1.300 \pm 0.01 | 1.279 \pm 0.01 |
| P | 1680 \pm 119 | 1714 \pm 41 | 1799 \pm 68 | 1751 \pm 40 ^a |
| Se | 54.6 \pm 16.8 | 107.3 \pm 28.3 ^e | 129.8 \pm 38.9 ^e | 60.9 \pm 10.9 ^{a,c} |
| S | 1273 \pm 22 | 1562 \pm 14 ^e | 1404 \pm 11 ^{a,e} | 1345 \pm 24 ^{a,c,e} |
| Zn | 14.79 \pm 0.04 | 14.33 \pm 0.02 | 13.96 \pm 0.12 ^a | 13.49 \pm 0.10 ^{a,c} |

[†] $P < 0.05$ vs sham operated group, ^a $P < 0.05$ vs hyperemia group, ^c $P < 0.05$ vs control group

Chemiluminescent intensity was significantly higher in the sham operated group ($1.339 \times 10^4 \pm 1.42 \times 10^4$ RLU) (as well as during hyperemia) than in the control group ($1.612 \times 10^7 \pm 1.03 \times 10^6$ RLU). Chemiluminescent intensity was normalized by reperfusion of the ligated lobes (Figure 1B). The concentration of diene conjugates as well as the chemiluminescent intensity pointed to oxidative damage to the liver tissue.

H-donating ability was lower in the hyperemia group (36.05 ± 0.12) than in the sham operated group (33.53 ± 2.85). The changes in reducing power (159.36 ± 5.76 versus 140.16 ± 13.68 $\mu\text{molAS/g}$ protein) and in the concentration of free SH-groups (0.649 ± 0.054 versus 0.579 ± 0.042 mmol/L) showed the same tendency, although the changes were not significant (Figures 2A, 2B, 2C), indicating a decrease in non specific antioxidant defence. In the reperfusion group these parameters showed enormous deviation, suggesting that the antioxidant system had to exert a great ability.

A positive correlation ($P < 0.05$) was found between the changes in H-donating ability, reducing power and the concentration of free SH-groups ($r = 0.81$ between H-donating ability and reducing power, $r = 0.82$ between H-donating ability and SH-groups and $r = 0.56$ between reducing power and SH-groups), indicating the parallel work of the antioxidant defence system.

The SOD activity (in arbitrary unit) of the liver decreased significantly in the hyperemia group (0.634 ± 0.133) compared to that in the sham operated group (1.073 ± 0.122) (Figure 2D). The glutathione-peroxidase (GSHPx) activity (in arbitrary unit) was significantly lower in the hyperemia group (263.11 ± 45.17) than in the sham operated group (380.70 ± 33.88). When the hepatic flow became normal, the activity of the enzyme approached the basic state (328.38 ± 51.87) (Figure 2E). The level of TAS (in arbitrary unit) was also significantly lower in the hyperemia group (1.936 ± 0.027) than in the sham operated group (2.214 ± 0.165) (Figure 2F).

The concentration of elements (Al, Ca, Cu, Fe, Mg, Mn, P, S, Se and Zn) was also determined from pooled liver samples by ICP-OES and polarography. The concentration of Al, Cu, Mn, Zn, and S was lower in the hyperemia

Table 2 Significant correlations between metal elements in liver during experiment

| Metal ions | Correlations | Metal ions | Correlations |
|------------|-----------------------|------------|-----------------------|
| Al-Fe | $r = -0.92; P < 0.05$ | P-Zn | $r = -0.73; P < 0.05$ |
| Ca-Fe | $r = 0.85; P < 0.05$ | P-S | $r = 0.99; P < 0.05$ |
| Ca-Mg | $r = 0.92; P < 0.05$ | Se-Cu | $r = 0.75; P < 0.05$ |
| Ca-S | $r = 0.86; P < 0.05$ | Se-Mn | $r = 0.83; P < 0.05$ |
| Ca-P | $r = 0.87; P < 0.05$ | Se-Ca | $r = 0.75; P < 0.05$ |
| Cu-Mn | $r = 0.72; P < 0.05$ | Se-Mg | $r = 0.95; P < 0.05$ |
| Mg-S | $r = 0.85; P < 0.05$ | Se-S | $r = 0.77; P < 0.05$ |
| Mg-P | $r = 0.79; P < 0.05$ | | |

group than in the sham operated group, while the level of Ca, Fe, Mg, Se and P ions increased during hyperemia. The tendency was the same in the case of Cu, Mn, Se and Zn level during reperfusion, which were essential for the activity of SOD and GSHPx enzymes (Table 1).

The correlations between the changes in metal ion content are listed in Table 2.

DISCUSSION

Hepatic overflow has not been studied thoroughly so far. The intact liver lobes of the rat ischaemia-reperfusion model are generally considered unharmed, whereas oxidative damage has been verified in our investigations. The injury is probably due to the generation of free radicals. The presence of oxidative stress causes disturbances in the cell cycle and initiates apoptosis^[27]. Hepatic apoptosis is related to the generation of interferon- γ , which is able to produce oxygen-related free radicals as well^[28].

The production of reactive oxygen substances leads to hepatocyte damage, therefore, poor liver function becomes even worse^[29]. A better understanding of these mechanisms is important, since the generation of free radicals and the decrease in antioxidant level can be compensated with scavenger molecules such as glutathione and multivitamin infusion^[30-32]. Moreover, aged patients after liver surgery have significantly weaker antioxidant defence than young ones^[33].

Compared to the control group, the level of diene conjugates and the chemiluminescent intensity were significantly higher in the sham operated animals as well as in the animals with liver overflow. The high level of lipid peroxidation is one of the markers of oxidative stress, which along with the changes in chemiluminescent intensity point to hepatocyte injury. Chemiluminescent intensity is considered as the amount of generated free radicals. The generation of free radicals is related to the production of cytokines and other inflammatory agents, and NF- κ -B is also activated in the process^[34], which finally leads to apoptosis and/or necrosis.

Apoptosis may occur in a physiological setting without

inflammation. In pathophysiological settings apoptosis frequently induces inflammation because of the onset of secondary necrosis and stimulation of cytokine and chemokine formation. In liver, mitochondrial permeability transition represents a shared pathway that leads to both necrosis and apoptosis (adenosine triphosphate depletion, cytochrome C release), which makes discrimination between the two forms of cell death difficult sometimes^[35].

Signal transducer and activator of transcription-3 (Stat3) are the most important molecules involved in the initiation of liver regeneration. Stat3 activation protects against Fas-mediated liver injury by inhibiting caspase activities in redox-dependent and -independent mechanisms^[36]. Stat3 provides hepatoprotection against Fas-mediated apoptotic liver damage by two mechanisms: direct inactivation of caspases and reduction of reactive oxygen species^[37].

During hepatic overflow the antioxidant system is injured as well. The non-enzymatic defence system can be described by determining the values of H-donating ability, reducing power and the concentration of free SH-groups, which were found to be lower in this experiment. When the defence activity is lower, free radicals induce severer damage, suggesting that these factors are of great importance. The changes in enzymatic defence (TAS, SOD, GSHPx) show the same tendency, as observed in non-enzymatic defence. These changes may be suitable markers of hepatocyte damage. The joint effect of the high amount of reactive oxygen species produced and the decrease in the defence system may be the key to a better understanding of the damage during hepatic hyperemia.

In the present study, the high amount of blood flow caused the transfer of some metal ions into the liver cells. Especially the Ca, Fe, Mg, and P content in the liver increased during hyperemia, which can be explained by the injury of cellular membranes. The amount of the generated diene conjugates also pointed to the injury of cellular membranes. The concentration of most of the essential metal ions increased in the liver of sham operated rats. The accumulation of Fe during hepatic overflow and after surgery is important, since this element can induce the production of free radicals through the Fenton reaction. A strong positive correlation was found between the changes in concentrations of Ca-Fe, Ca-Mg, Ca-S, Ca-P, Cu-Mn, Mg-S, Mg-P, and P-S, while there was a strong negative correlation between the levels of Al-Fe and P-Zn. The accumulation of certain elements in the liver during surgery may be harmful.

Since the presence of Mn, Zn and Cu is essential for the function of SOD enzymes, their lower ability may be attributed to the loss of the above elements^[38]. Se is relevant to the action of glutation-peroxidase, which is also important in the antioxidant defence of liver cells. During our experiment the behaviour of Se was surprising. Therefore, measuring Se concentration under these conditions may lead to a new and important field of research^[39]. These findings also underline the changes in the homeostasis of liver cells due to hyperperfusion.

Compared to the ischaemia-reperfusion damage^[40], the injury caused by hyperemia is related to less hepatocyte dysfunction, an important risk factor for liver surgery. In conclusion, the methods applied by us are suitable for

measuring compensatory mechanisms in hyperemia and ischaemia-reperfusion.

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BASIC RESEARCH

Cloning and characterization of porcine aquaporin 1 water channel expressed extensively in gastrointestinal system

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permeability that is inhibitable by HgCl₂ was detected in porcine erythrocytes and CHO cells stably transfected with pAQP1 cDNA. Immunoblot analysis of porcine erythrocytes and pAQP-transfected CHO cells revealed an unglycosylated 28 ku band and larger glycosylated proteins.

CONCLUSION: pAQP1 is the first porcine aquaporin that can be molecularly identified so far. The broad distribution of pAQP1 in epithelium and endothelium of porcine digestive organs may suggest an important role of channel-mediated water transport in fluid secretion/absorption as well as in digestive function and pathophysiology of the gastrointestinal system.

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Key words: Aquaporin; Molecular cloning; Porcine gastrointestinal organs; Water transport; Digestive function

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Abstract

AIM: To clone and characterize the porcine aquaporins (AQPs) in the gastrointestinal system.

METHODS: A PCR-based cloning strategy and RACE were used to clone full-length AQP coding sequence from reversely transcribed pig liver cDNA. Stopped-flow light scattering and a YFP-based fluorescence method were used to measure the osmotic water permeability of erythrocytes and the stably transfected CHO cells. RT-PCR, Northern blot, and immunohistochemistry were used to determine the gastrointestinal expression and localization of cloned AQPs. Protein expression in transfected cells and red blood cells was analyzed by Western blot.

RESULTS: An 813 bp cDNA encoding a 271 amino acid porcine aquaporin (designated pAQP1) was cloned from liver mRNA (pAQP1 has a 93% identity with human AQP1 and contains two NPA motifs conserved in AQP family, one consensus sequence for N-linked glycosylation, and one mercury-sensitive site at cysteine 191). RT-PCR analysis revealed extensive expression of pAQP1 mRNA in porcine digestive glands and gut. Northern blot showed a single 3.0 kb transcript in selected digestive organs. pAQP1 protein was localized at central lacteals of the small intestine, microvessles of salivary glands, as well as epithelium of intrahepatic bile ducts by immunoperoxidase. High osmotic water

INTRODUCTION

Aquaporins (AQPs) are water channel proteins located on membranes of various cell types where they create high water permeability. So far at least 12 mammalian members of AQP family have been molecularly localized in diverse fluid transporting tissues have been molecularly identified. Recent studies on human subjects with AQP gene mutations using transgenic AQP knockout mice indicate that AQPs have important functions in multi-tissue physiology and pathophysiology^[1,2]. In the gastrointestinal (GI) system, several aquaporins (including AQPs 1, 3-5, 7-10) are localized on various epithelial and endothelial cell membranes of human and rodent GI organs and provide a trans-cellular pathway for fast water movement during fluid secretion and absorption^[3-6]. Studies on AQP knockout mice demonstrated that AQP5 plays a role in saliva secretion^[7], AQP1 a role in dietary fat processing^[8], and AQP4 a role in colonic fluid absorption and fecal dehydration^[9]. On the other hand, localization of AQPs

in some mouse GI organs does not indicate physiological importance. For example, AQP4 deletion in gastric parietal cells does not affect gastric acid secretion^[10], AQP1 deletion in micro-vessels of salivary glands does not affect saliva secretion, AQP1 deletion in intra-hepatic bile ducts does not affect the flow rate and components of bile^[8]. GI phenotype in AQP1-null human subjects has not been reported so far, which may indicate species differences of AQP involvement in digestive physiology.

Although significant progresses have been made in molecular biology and physiology of AQP family in the GI system, identification and characterization of AQPs in GI organs of mammalian species other than in those of human and rodents have been poorly studied. The pig GI system more resembles the human GI system both structurally and physiologically in model animals^[11]. However, no porcine AQP member has been identified molecularly so far. In the present study, we cloned the first porcine aquaporin, pAQP1 from pig liver, by a PCR-based homologous cloning strategy. Its functional properties and distribution in pig GI system were analyzed.

MATERIALS AND METHODS

cDNA cloning of pAQP1

Full-length coding sequence of pig AQP1 cDNA was cloned using a PCR-based homologous cloning strategy^[12]. Total RNA was extracted from pig liver using TRIZOL reagent (Invitrogen). mRNA was isolated from the total liver RNA using an Oligotex mRMA kit (Qiagen). cDNA was reversely transcribed from the mRNA using a first strand cDNA amplification kit (Invitrogen) and used as template for 30 cycles of PCR amplification at 94 °C for 30 s, at 55 °C for 30 s, at 72 °C for 1 min with degenerate oligonucleotide primers designed according to amino acid sequences around the two NPA motifs of aquaporin family: sense: 5'-CA(C-T)IT(CA)AA(CT)CCIGCIGTIAC-3'; antisense: 5'-(G C) C I (A G) (A G) I (A - G)(AT)GC(TG)IGC(AT)GG(AG)TT-3'. PCR products of about 360 bp were subcloned into pCR2.1TOPO TA cloning vector (Invitrogen) and sequenced. A sequence with open reading frame that is most homologous to dog AQP1 water channel was identified. To clone the full length coding sequence of the candidate water channel, 5'- and 3'-RACE (rapid amplification of cDNA ends) were performed using the Marathon cDNA amplification kit (BD Biosciences-Clontech) following the provided protocol. The sequences of gene-specific primers (GSP) are as follows: 5'-RACE GSP: 5'-CACACACTGGGCAA TGATGTACATG-3'; 5'-RACE NGSP: 5'-GACACTGA TCTGGCAGCTGAGCAG-3'; 3'-RACE GSP: 5'-CCTT GCCATCGGCTTCTCTGTGGC-3'; 3'-RACE NGSP: 5'-CTGGGACACCTGCTGGCGATTGAC-3'. An Expond long PCR kit (Roche) was used for 30 cycles of RACE amplification at 94 °C for 30 s, at 65 °C for 30 s, at 68 °C for 4 min. PCR products were subcloned to pCR2.1TOPO TA cloning vector and sequenced. The longest open reading frame was obtained by joining the 5'-RACE and 3'-RACE sequence and the sequence between the two NPA motifs. The protein encoded by the longest open reading frame was designated as pAQP1.

RT-PCR and Northern blot

Total RNA was extracted from pig salivary glands, liver, pancreas, esophagus, stomach, small intestine and colon respectively, using TRIZOL reagent. For RT-PCR analysis, cDNAs were reversely transcribed from 5 µg of each total RNA using first strand cDNA amplification kit and 30 cycles of PCR amplification were performed at 94 °C for 30 s, at 62 °C for 30 s, at 72 °C for 2 min with primers flanking the coding sequence of pAQP1 (sense: 5'-CG-GATCCATGGCCAGCGAGTTCAAGAAG-3'; antisense: 5'-GCTCTAGATTATTTGGGCTTCATCTCCACC-3'. For Northern blot, total RNA (20 µg) from liver, salivary gland, small intestine and colon was resolved on 1.2% formaldehyde gel and transferred to a Hybond-N⁺ nylon membrane (Amersham). The membrane was prehybridized at 68 °C for 1 h in a rapid hybridization buffer (Amersham) and then hybridized for 1 h with a ³²P-labeled probe corresponding to the full-length pAQP1 coding sequence. The membrane was washed three times in 0.2 x SSC, 0.1% SDS at 68 °C, each for 15 min, and autoradiographed for 16 h with double intensifying screens. The membrane was boiled in water and re-probed with a 600 bp pig β-actin cDNA sequence.

Transfection and water permeability measurement of pAQP1 in CHO cells

A cell-based fluorescence microassay employing a Cl⁻-sensitive EYFP mutant^[13] was developed to measure the channel-mediated water permeability of plasma membrane. The 720 bp EYFP-H148Q-V163S DNA fragment encoding the Cl⁻-sensitive EYFP mutant was PCR-amplified using Vent DNA polymerase (New England Biolabs) and primers with engineered restriction sites *Hind*III (5' primer) and *Xba*I (3' primer). The fragment was digested with *Hind*III/*Xba*I and ligated to the mammalian expression vector pcDNA3.1 Hygro (hygromycin-resistant, Invitrogen) predigested with *Hind*III/*Xba*I to form expression plasmid pcDNA3.1 Hygro-EYFP-H148Q-V163S. For pAQP1 expression, the 813 bp pAQP1 full length coding sequence was PCR-amplified using Vent DNA polymerase and primers with engineered restriction sites *Bam*HI (5' primer) and *Xba*I (3' primer). The amplified pAQP1 fragment was digested with *Bam*HI/*Xba*I and ligated to the mammalian expression vector pcDNA3.1(+) (G418-resistant, Invitrogen) predigested with *Bam*HI/*Xba*I to form expression plasmid pcDNA3.1 pAQP1.

CHO-K1 cells were first transfected with expression plasmid pcDNA3.1Hygro-EYFP-H148Q-V163S using Lipofectin reagent (Invitrogen). After selection by hygromycin (Roche) at 500 µg/mL, highly expressed CHO clones were isolated by judging brightness of survived cell colonies under fluorescence microscope. The YFP-expressing cell clone, CHO-K1/EYFP-H148Q-V163S, was then transfected with pcDNA3.1 pAQP1 and selected with G418 (Invitrogen) at 500 µg/mL. pAQP1 expression in survived CHO cell clones was analyzed by immunoblot. CHO cell clones with high cytoplasmic expression of Cl⁻-sensitive EYFP mutant and plasma membrane expression of pAQP1 were selected for water permeability measurement.

A ATGGCCAGGAGTTCAAGAAGAGATCTTCTGGAGGGCGGTGGTGGCCGAGTTCCTGGCC 60
M A S E F K K K I F W R A V V A E F L A 20
ATGACCCCTCTCATCTTTCATCAGCATGGTTCTGCOCTGGGCTTCAGTACCCOOSTGAGG 120
M T L F I F I S I G S A L G F Q Y P V R 40
AACACCCAGACTTCAGGTGGGCGCCAGGATAAGCTGAAGGTGTGGCTGGCCCTTTGGGCTG 180
N N A Q T S G A A Q D N V K V S L A F G L 60
AGCATTGCCAGCTGGCCAGAGAGTGGGCCACATCAGCGGGGCGCCACTCAACCCGGCC 240
S I A T L A Q S V G H I S G A H L N P A 80
GTCAGGCTGGGCTCTGCTCAGCTGOCAGATCAGTGTCTCOGGGCTGTCATGTATATC 300
V T L G L L L S C Q I S V L R A V M Y I 100
ATTGCCAGTGTGGGGGCGCATGTCGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 360
I A Q C V G A I V A T A I L S G I T S S 120
CTGCCCGCACTCOCTGGGCTGAATCGCTGGCCOCTGGGCTGGACTGGGCCAGGGC 420
L F G N S L G L N S L A P G V D S G Q G 140
CTGGGATGGAGATCATTGGCACCCCTGCAGCTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGT 480
L G I E I I G T L Q L V L C V L A T T D 160
AGGAGGCGCGGGACCTTGGGGCTCTGCCOCCCTTGCATCGGCTTCTCTGTGGCCCTG 540
R R R R D L G G S A P L A I G F S V A L 180
GGACACTGCTGGGCGATTGACTACACGGCTGGGGCATTAAOCTGCOOCTGCTTGGG 600
G H L L A I D Y T G C G I N P A R S F G 200
TCOOGGTGATCAGCACAACCTCCAGGACACTGGGTTTCTGGGTGGGCGOCTTTCATC 660
S A V I T H N F Q D H W V F W V G P F I 220
GGAGGCGCTGGGCGTCTATGACTTCACTGCTGGGCGCGCAGCAGCAGCAGCT 720
G G A L A V L I Y D F I L A P R S S D L 240
ACAGACCGGTGAAGGTGTGGACGGCCAGGTGGAGGATGACTGGATGGCGAC 780
T D R V K V W T S G V E E Y D L D G D 260
GACATCACTCCAGGTTGGAGATGAAGCCCAATAA 816
D I N S R V E M K P K Z 271

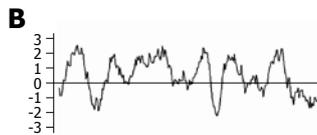


Figure 1 cDNA and deduced amino acid sequences of pig AQP1 water channel. **A:** Two NPA sequences conserved in water channel proteins of aquaporin family (in bold). * indicates the consensus sequence for N-linked glycosylation and the mercurial-sensitive site cysteine 201 near the second NPA motif is underlined; **B:** Kyte-Doolittle hydropathy profile of the deduced pAQP1 amino acid sequence.

For water permeability measurement, the CHO-K1/EYFP-H148Q-V163S/pAQP1 cells were plated in 96-well clear bottom of black-walled microplates (Costar) at the density of 20000 cells per well in F-12 Ham's medium supplemented with 10% FBS and 0.5 mg/mL G418. After incubation at 37 °C in an atmosphere containing 5% CO₂ with 90% humidity for 18 h, the cells in 96-well plates were washed twice in PBS buffer (200 μL/wash), leaving 100 μL PBS after the last wash. Measurement was performed on FLUOstar Optima plate reader (BMG Technology) equipped with syringe pumps and HQ500/20X (500 ± 10 nm) excitation and HQ535/30M (535 ± 15 nm) emission filters. Cells in each well of the plate were assayed individually for osmotically driven pAQP1-mediated water influx across the plasma membrane that dilutes the cytoplasmic Cl⁻ by recording fluorescence increase continuously (0.2 s per point) for 2 s (baseline) and then for 21 s (water transport into the cells) after rapid injection (< 1 s) of 100 μL distilled water. Water permeability was expressed as half time (t_{1/2}) needed from water injection to the point when the cytoplasmic fluorescence reached the maximum. The smaller t_{1/2} represented the higher water permeability.

Measurement of erythrocyte water permeability

Pig blood was drawn from the ear by venipuncture, and the washed erythrocytes were diluted into phosphate buffered saline (PBS) at a 0.1 vol %. Osmotic water permeability was measured by a stopped-flow light scattering technique in which the diluted erythrocyte suspension was mixed rapidly with PBS containing 100 mmol/L sucrose^[14]. Erythrocyte volume was measured continuously by 90 s light scattering for 90 s at a 520-nm wavelength.

Immunohistochemistry

Paraffin-embedded blocks were prepared from fresh normal pig salivary gland, liver, and small intestine fixed in 4% formalin. For immunohistochemistry, 3-μm sections of pig tissues were prepared by standard procedures and exposed to the primary affinity-purified rabbit-antirat AQP1 antibody (AB3065, Chemicon International) diluted at 1:1000 for 1 h at room temperature. In some experiments, the primary AQP1 antibody was preincubated with the immunizing peptide before administered to the tissue sections. After washed, the sections were exposed to a horseradish peroxidase-conjugated goat antirabbit IgG (A6154, Sigma) secondary antibody at 1:2000 dilution followed by development with 3,3'-diaminobenzidine (DAB) liquid substrate dropper system (D7679, Sigma). The sections were counterstained with haematoxylin as the last step.

Western blotting

Stably transfected CHO cells were lysed in 10 mM HEPES buffer containing 0.5% SDS, 100 mmol/L DTT and 57.4 μmol/L PMSF (pH 7.5). Red blood cells from pigs, human beings and mice were first burst in hypoosmotic solution, and then the ghosts were centrifuged and lysed in SDS buffer. Protein concentrations in CHO cell and ghost lysates were determined spectrophotometrically. For immunoblotting, 20 μg proteins from the CHO cell lysate or 5 μg proteins from the ghost lysate of red blood cells from pigs, human beings and mice were dissolved in protein sample buffer, heated at 65 °C for 10 min, and then resolved on 12% SDS-PAGE minigels. Proteins were blotted into PVDF membranes, blocked for 1 h, washed with TBS-T (pH 7.4), and incubated for 1 h at room temperature with anti-AQP-1 antibody diluted at 1:1000. After washed, membranes were incubated with HRP-conjugated goat anti-rabbit IgG (1:3000, Sigma), and immunoreactive sites in membranes were revealed by enhanced chemiluminescence (Amersham).

RESULTS

Cloning and analysis of pAQP1 cDNA

The full-length cDNA coding sequence and the predicted amino acid sequence of pAQP1 (Genbank accession no. AY585335) are shown in Figure 1A. The 813 bp open reading frame encoded a 271-amino acid protein containing the two conserved NPA motifs found in most AQP family members and a cysteine residue located just upstream of the second NPA motif, which could confer mercurial sensitivity in several cloned mammalian AQPs^[15,16]. Compared to human and mouse AQP1 coding sequences, an additional segment of GCGGCC at nt 138–143 encoding tow alanines (residues 47 and 48) was found only in the porcine sequence. Figure 1B shows the Kyte-Doolittle hydropathy plot for pAQP1, suggesting six membrane spanning domains similar to those of human and rat AQP1. Figure 2 shows an amino acid sequence alignment of pAQP1 with its homologues from dogs, human beings, and mice. pAQP1 had the greatest amino acid identity to dog AQP1 (94%) and high homology to human AQP1 (93%) and mouse AQP1 (91%). The

| | |
|-------|--|
| Pig | MASEFKKKIFWRAVVAEFLAMTLFIFISIGSALGFQYPRVNNQTSAGAAQDNVKVSLAFGL 60 |
| Dog | MASEFKKLFWRVVAEFLAMI LFFVIFISIGSALGFNYPVRNNQTAGAAQDNVKVSLAFGL 60 |
| Human | MASEFKKLFWRVVAEFLAT T LFFVIFISIGSALGFKYVPGNNQTTV - - QDNVKVSLAFGL 58 |
| Mouse | MASEIKKLFWRVVAEFLAMTLFFVIFISIGSALGFNYPLE RNQTLV - - QDNVKVSLAFGL 58 |
| | |
| Pig | S IATLAQSGVGHISGAHINPAVTLGLLLSQCISILRAVMYII AQCVGAI VATA ILSGI TSS 120 |
| Dog | S IATLAQSGVGHISGAHINPAVTLGLLLSQCISILRAVMYII AQCVGAI VATA ILSGI TSS 120 |
| Human | S IATLAQSGVGHISGAHINPAVTLGLLLSQCISIFRALMYII AQCVGAI VATA ILSGI TSS 118 |
| Mouse | S IATLAQSGVGHISGAHINPAVTLGLLLSQCISILRAVMYII AQCVGAI VATA ILSGI TSS 118 |
| | |
| Pig | LPGNSLGLNSLAPGVDSGQGLGIE IIGTLQLVLCVLAT TDR RRRDLGGSAPLA IGLSVAL 180 |
| Dog | LPGNSLGRNE LAPGVNSGQGLGIE IIGTLQLVLCVLAT TDR RRRDLGGS P LA IGLSVAL 180 |
| Human | LTGNSLGRNDLADGVNSGQGLGIE IIGTLQLVLCVLAT TDR RRRDLGGSAPLA IGLSVAL 178 |
| Mouse | LVDNSLGRNDLAHGVNSGQGLGIE IIGTLQLVLCVLAT TDR RRRDLGGSAPLA IGLSVAL 178 |
| | |
| Pig | GHL LAI DYTGG I NPA R S F G S AVI THNFQDHWVFWGPF I GGALAVL I YDF I LAPRSSDL 240 |
| Dog | GHL LAI DYTGG I NPA R S F G S SVI THNFQDHWI FWGPF I GGALAVL I YDF I LAPRSSDL 240 |
| Human | GHL LAI DYTGG I NPA R S F G S AVITHNFSNHVI FWGPF I GGALAVL I YDF I LAPRSSDL 238 |
| Mouse | GHL LAI DYTGG I NPA R S F G S AVLTRNFSNHVI FWGPF I GGALAVL I YDF I LAPRSSDF 238 |
| | |
| Pig | TDRVKVWTSQGV EEYDLGDGDD INSRVEMKPK 271 |
| Dog | TDRVKVWTSQGV EEYDLGDGDD INSRVEMKPK 271 |
| Human | TDRVKVWTSQGV EEYDLGDADD INSRVEMKPK 269 |
| Mouse | TDRMKVWTSQGV EEYDLGDADD INSRVEMKPK 269 |

Figure 2 Amino acid sequence alignment of pAQP1 with its dog, human and mouse orthologs. Conserved amino acids are shielded, NPA motifs are boxed and cyteines near the second NPA motifs are underlined. The consensus N-linked glycosylation sites are shown in bold.

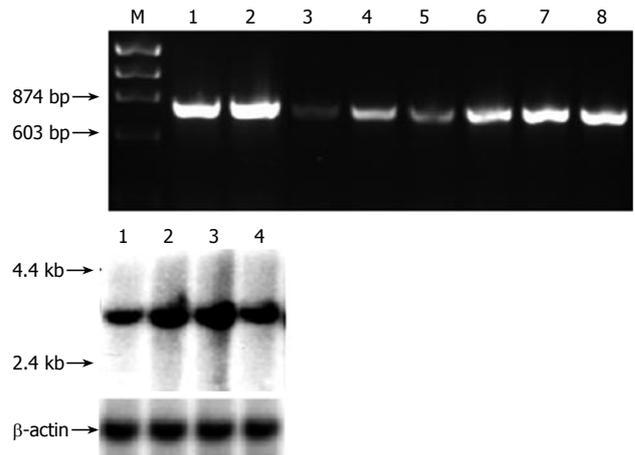


Figure 3 mRNA expression of pAQP1 water channel in pig gastrointestinal organs. **A:** Expression of pAQP1 transcript by RT-PCR analysis. Lanes 1, Salivary gland; 2, Liver; 3, Pancreas; 4, Esophagus; 5, Stomach; 6, Jejunum; 7, Ileum; 8, Colon; **B:** Northern blotting of total RNA (20 µg) from salivary gland (lane 1), liver (lane 2), ileum (lane 3) and colon (lane 4) probed with the pAQP1 coding sequence (upper) and the same membrane probed with a 600 bp pig β-actin cDNA sequence (lower).

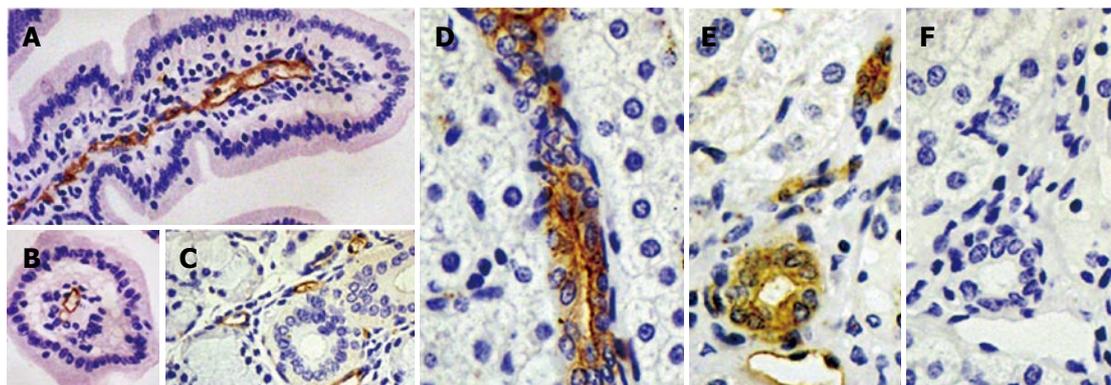


Figure 4 Immunolocalization of pAQP1 in pig gastrointestinal tract and exocrine glands. Specific pAQP1 labeling of central lacteals was seen by immunoperoxidase staining of longitudinal (A) and traverse (B) sections of the small intestinal villi using an affinity-purified AQP1 antibody. Arrows indicate the endothelium of central lacteals; A salivary gland section showing specific labeling of microvessel endothelium indicated by arrows (C); Specific pAQP1 labeling was seen in the epithelium of intrahepatic bile ducts in longitudinal (D) and traverse (E) sections. Arrows indicate heavy pAQP1 staining in the apical domain of bile duct epithelial cells. Arrowhead indicates pAQP1 labeling in the endothelium of periductal microvessels; A consecutive section of E showing immunostaining with AQP1 antibody preabsorbed with the immunizing peptide (F).

pAQP1 had a 29 - 52% similarity in amino acid sequences to other mammalian aquaporin members^[15, 16]. There were two consensus sequences for phosphorylation by protein kinase-C like in human and mouse AQP1. However, pAQP1 contained only one consensus site at residue 42 for N-linked glycosylation compared to two sites in human and mouse AQP1 sequences at residues 42 and 205. In addition, there were two more amino acids in the pig and dog AQP1 sequences at residues 47 and 48 instead of in the human and mouse sequences.

Expression and immunolocalization of pAQP1 in digestive organs

Multitissue RT-PCR analysis indicated broad expression of pAQP1 mRNA in pig digestive organs including salivary glands, pancreas, liver and different segments of the gut (Figure 3A). Figure 3B shows Northern blot analysis of total RNA from pig salivary glands, liver, small intestine, and colon respectively using the full length pAQP1 cDNA

as a probe. A single transcript of about 3.0 kb was seen in liver ~ small intestine > colon > salivary glands. Figure 4 shows an immunoperoxidase staining of pAQP1 in selected digestive organs. Specific AQP1 labeling was seen both in the endothelia of central lacteals in villi of the small intestine (Figures 4A and 4B) and in the endothelia of microvessels in salivary glands (Figure 4C). In the liver, AQP1 staining was mainly seen both in the epithelium of small bile ducts and endothelium of some small vessels (Figures 4D and 4E). AQP1 labeling was predominant in the apical domain of most stained bile ducts.

Functional characterization of pAQP1

Since AQP1 is a erythrocyte water channel, osmotic water permeability of pig red blood cells was analyzed using stopped-flow light scattering and compared with human and mouse red blood cells. Figure 5A shows the time course of osmotic cell shrinking in response to a 100 mmol/L inwardly directed osmotic gradient.

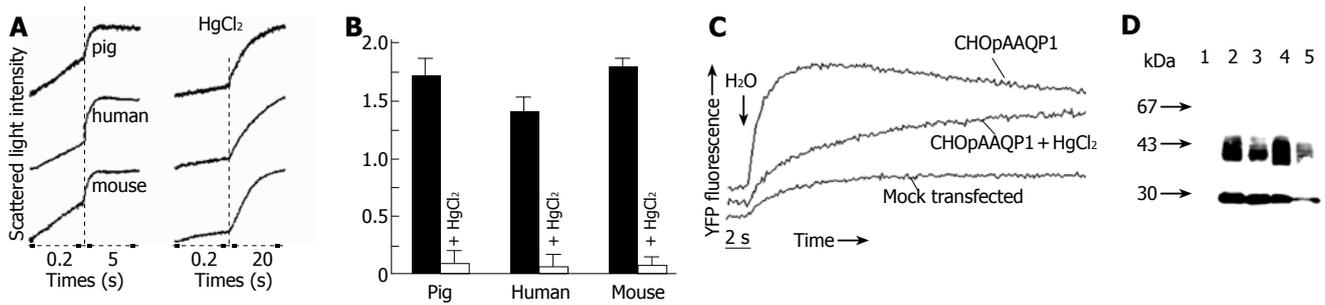


Figure 5 Functional properties of pAQP1 in erythrocytes and stably transfected CHO cells. **A:** Osmotic water permeability of pig, human and mouse erythrocytes measured by stopped-flow light scattering from the time course of erythrocyte volume in response to a 100 mmol/L inwardly directed sucrose gradient in the absence (left panel) and presence (right panel) of 0.3 mmol/L HgCl₂; **B:** Summary of osmotic water permeability coefficient (Pf) for erythrocytes from pig, human and mouse measured in A (mean ± SE, n=4); **C:** Osmotic water permeability of CHO cells stably transfected with pAQP1 cDNA measured by YFP-based fluorescence assay. Water permeability of CHO cells was expressed as half time (t_{1/2}) needed from water injection to the point when the cytoplasmic fluorescence reached the maximum. t_{1/2} of pAQP1 transfected CHO cells in the absence and presence of 0.1 mmol/L HgCl₂ was 1.2 s and 4.6 s separately. t_{1/2}>20 s in mock-transfected CHO cells; **D:** Immunoblot of erythrocytes and transfected CHO cells. Lanes 1-4: erythrocytes from AQP1^{-/-} mice, AQP1^{+/+} mice, pigs and human beings. Lane 5: CHO cells stably transfected with pAQP1 cDNA.

Two contiguous time scales were used to plot the full time course of decreasing cell volume (increasing light intensity). The computed osmotic water permeability of pig, human and mouse erythrocytes is summarized in Figure 5B. The pig erythrocyte membrane showed a high osmotic water permeability that was inhibited > 90% by 0.3 mmol/L HgCl₂, similar to that of human and mouse erythrocytes. Water channel function of pAQP1 was also analyzed in heterologous expression system. Figure 5C shows the time course of YFP fluorescence of CHO cells stably transfected with pAQP1 cDNA in response to extracellular hypotonicity (50% PBS). The t_{1/2} of fluorescence dynamics for pAQP1-transfected cells was 1.2 s compared to 4.6 s for the same cells incubated with 0.1mmol/L HgCl₂ and > 20 s for mock-transfected cells, indicating that a high membrane water permeability was mediated by pAQP1 water channel inhibited by mercurial compounds. Figure 5D shows Western blot analysis of red blood cells and pAQP1-transfected CHO cells using an affinity-purified AQP1 antibody. An unglycosylated 28 ku band and glycosylated proteins at a higher molecular weight were determined both in red blood cells of human beings, pigs, wildtype mice and in pAQP1-transfected CHO cells, but missed in AQP1-knockout mouse erythrocytes. There was a significant decrease of glycosylated protein in pAQP1 compared to human AQP1 and mouse AQP1 in red blood cells. Western blot analysis of pig gastrointestinal tissues was not done because of the difficulty to remove red blood cell contamination.

DISCUSSION

We have successfully cloned pAQP1, the first molecularly identified porcine AQP water channel. The amino acid sequence of pAQP1 has a high identity to dog, human and mouse AQP1 and contains the two highly conserved NPA motifs and the mercurial-sensitive cysteine residue before the second NPA motif in several cloned AQPs in other mammals^[16-19]. pAQP1 contains only one consensus sequence compared to two consensus sequences for N-linked glycosylation in human and mouse AQP1^[20, 21]. Immunoblot analysis showed that glycosylated protein of pAQP1 was decreased compared to human

and mouse AQP1 in red blood cells, indicating that the second consensus site at asparagine 205 of human and mouse AQP1 contributes significantly to the N-linked glycosylation of the protein.

Like its human and mouse orthologs, pAQP1 conferred a high osmotic water permeability in a mercurial-sensitive manner both in pig red blood cells and in heterologous expression system, indicating a similar functional water channel in erythrocyte and somatic cell membranes. pAQP1 mRNA and protein were expressed extensively in endothelia and epithelia of pig gastrointestinal system involving digestive glands responsible for secretion of saliva, bile, and pancreatic juice as well as all the intestinal segments responsible for absorption of fluids and nutrients, suggesting that channel-mediated transcellular water pathway plays a role in broad physiological processes of secretion and absorption in various porcine digestive organs. The molecular cloning of pAQP1 makes it possible to investigate its expression and regulation during development and in various physiological and pathophysiological conditions of the pig gastrointestinal system.

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RAPID COMMUNICATION

Therapeutic effectiveness of echo-guided percutaneous radiofrequency ablation therapy with a LeVeen needle electrode in hepatocellular carcinoma

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Abstract

AIM: To investigate the results of radiofrequency ablation (RFA) in obtaining the necrosis of hepatocellular carcinoma (HCC) in cirrhotic patients and to assess the results of RFA in relation to recurrence of HCC and survival of the treated patients.

METHODS: Fifty-six consecutive cirrhotic patients with 63 HCCs were treated with RFA between May 2000 and May 2004. The diameter of the HCCs ranged from 1 cm to 5 cm (mean 2.8 cm). In all cases RFA was performed with percutaneous approach under ultrasound guidance using expandable needle electrode (LeVeen needle). Treatment efficacy and recurrence were evaluated with dual-phase spiral computed tomography (CT).

RESULTS: Complete necrosis after single or multiple treatment was achieved in 96.8% (61/63) tumors. We observed recurrence after complete necrosis in 23 patients (41%) during a mean follow-up of 32.3 months. The recurrences were local in 2 patients (8.6%) and in different segments in 21 (91.4%). Major complications occurred in 3 patients (4%). During follow-up period, 32 (57.1%) patients died; 15 due to progression of HCC, 11 from liver failure, 3 from esophageal varices bleeding and 3 from the causes not related to liver disease.

CONCLUSION: RFA with LeVeen needle is an effective and safe treatment for HCC < 5 cm in cirrhotic patients. It has yet to be established how far this treatment influences the survival rate of patients. It becomes important to establish treatments to prevent recurrences in different segments, such as interferon therapy.

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Key words: Hepatocellular carcinoma; Radiofrequency ablation; Complication; Recurrence

INTRODUCTION

Hepatocellular carcinoma (HCC) is a common malignant neoplasm throughout the world and surgical resection is thought to be the best curative approach in the treatment of HCC^[1-3]. However, few patients with HCC are suitable for resection due to age, location of the lesion, advanced cirrhosis of the liver and concomitant diseases; as a consequence less invasive treatment methods have been pursued, especially echo-guided percutaneous ethanol injection (PEI) and radiofrequency (RF) thermal ablation^[4-9]. PEI was the first method used starting in the eighties, followed by the introduction of RF in order to overcome some of the limitations of PEI, such as the difficulty in ensuring the distribution of ethanol throughout the lesion and the need for multiple sessions due to the reduced volume of necrosis that can be achieved in the single session^[10]. At present RF is certainly the first choice of interstitial therapy method in the majority of cases of HCC that can not be treated surgically. The two types of RFA needles most used are the cool-tip needle with internally circulating cool saline (Radionics, Burlington, MA, USA) and the expandable 10-hooks LeVeen needle (Radio Therapeutics Corporation, Sunnyvale, CA, USA). Compared with the Cool-Tip, the LeVeen needle electrode can theoretically create a more even flux of the radiowave, producing thermal effects with a greater spherical area. Because this needle uses a gradual augmentation sequence of the RF output to prevent excess heating of the target tissue, this RFA system does not require circulation of cool saline in the needle electrode for preventing charring of the target tissue as in the Cool-Tip RFA system^[9]. The LeVeen needle has a larger diameter than the Cool-Tip (15 Gauge vs 18 Gauge), but no significant differences in complications have been reported in the literature between the two types of needle electrodes^[11].

Herein, we report our experience in performing

percutaneous echo-guided RF thermal ablation of HCCs with the LeVeen needle electrode system in a gastroenterological Italian service. We aimed to assess the efficacy of the system in obtaining the necrosis of the target tissue and the clinical outcome of the treated patients.

MATERIALS AND METHODS

From May 2000 to May 2004, a total of 56 consecutive cirrhotic patients (37 males and 19 females, age range 45-81 years, mean age 68.7 years), who underwent RFA for non-surgically resectable HCC due to the location of the tumor, severity of the cirrhosis, age, associated diseases or refusal of surgery, were enrolled in the study. Inclusion criteria were HCC diameter < 5 cm, absence of portal thrombosis or extrahepatic diffusion, absence of significant ascites, prothrombin activity > 50% and platelet count more than 50 000, and a maximum of three HCCs, each not more than 3 cm in maximum diameter.

The etiology of the cirrhosis was viral in 51 cases (48 due to HCV infection and 3 due to HBV infection), alcoholic in 4 and autoimmune in 1 case (primary biliary cirrhosis). On the basis of the Child-Pugh classification, 16 patients were class A, 37 class B and 3 class C (Table 1).

As regards the characteristics of the HCCs, 50 patients had a single tumor, 5 had two tumors and 1 had three tumors; a total of 63 tumors were detected in the 56 patients studied. None of the HCCs had an infiltrating appearance at imaging. The dimensions of the HCCs were between 1 and 2 cm in 19 cases, between 2 and 3 cm in 27 cases, between 3 and 4 cm in 12 cases and between 4 and 5 cm in 5 cases; the mean size was 2.8 cm (Table 2). The pre-treatment diagnosis of the nature of the lesion was histological with echo-guided fine needle biopsy in 42 patients, performed in 19/19 nodules <2 cm, 20/27 nodules <3 cm, 3/12 nodules <4 cm and 0/5 nodules <5 cm. In the remaining 14 patients, the diagnosis was based on non-invasive criteria, radiological with typical arterial hypervascularization at color power Doppler ultrasound, contrast-enhanced ultrasound, computerized tomography (CT) or magnetic resonance (MR) (11 patients) or combined with one typical imaging technique associated with AFP levels >400 ng/mL (3 patients). Starting in October 2000, as far as the non-invasive diagnosis was concerned, we followed the indications of the EASL Conference on clinical management of hepatocellular carcinoma, held in Barcelona, Spain in September 2000^[12]. It should however be pointed out that all the patients underwent pre-treatment contrast dual-phase spiral CT for the staging and baseline assessment of the vascularization of the HCC.

Technique

The RF delivery system was an RF 2000 generator system (Radio Therapeutics) in monopolar mode, used in conjunction with a LeVeen monopolar array needle electrode and two large dispersive electrodes. The RF generator supplies voltage at a frequency of 460 KHz and a maximum power output of 100 W. The RF generator display indicates power, tissue impedance and procedure

Table 1 Characteristics of 56 cirrhotic patients with HCC treated with RFA

| Parameters | Numbers |
|---------------------------|---------|
| Total patients | 56 |
| Gender | |
| Male | 37 |
| Female | 19 |
| Age (yr) | |
| Range | 45-81 |
| Mean | 68.7 |
| Type of cirrhosis | |
| Viral HCV-related | 48 |
| Viral HBV-related | 3 |
| Alcoholic | 4 |
| Autoimmune | 1 |
| Child-Pugh classification | |
| A | 16 |
| B | 37 |
| C | 3 |

time. The active expandable needle electrodes have a 15 gauge, 15 cm long stainless steel insulated cannula with 10 retractable lateral hooks each oriented at an angle of 36° from the two adjacent hooks. The maximum deployment diameter of the hooks was 3.0 or 3.5 cm. We used the 3.0 cm hooks for lesions equal to or less than 3 cm in diameter and the 3.5 cm hooks for lesions greater than 3 cm.

Treatment was performed only in inpatients, fasting 12 h before treatment, and they received mild sedation with an intramuscular administration of 10 mg of diazepam and 0.5 mg of atropine sulphate 30 min before the procedure. If necessary during treatment, 40 to 50 mg of pethidine was administered intravenously for analgesic purposes.

Using a multifrequency convex probe, RF ablation was performed with real-time ultrasound guidance (HID 3000, ATL, Bothell, WA, USA) by the same gastroenterologist (L.S.) with longstanding experience in interventional ultrasound. The needle was inserted using free-hand technique with subcostal or intercostal approach depending on the easiest access route according to the ultrasonographic picture. Once the needle electrode was located in the correct position, the hooks were deployed and the RF generator was activated. After maintaining the baseline power output at 50 W for 1 min, the output was increased by 10 W every minute until the power output reached 90 W, and then it was kept at 90 W until "roll-off" occurred. After a 20 to 60 s pause, power was reapplied at 75% of the maximum power achieved until power "roll-off" again occurred. If the "roll-off" did not occur within 15 min of the RF power output application, the RF power stopped automatically. After a 20 to 60 s pause, power was reapplied following the same procedure as before. Hematologic assays, such as red blood cell count and hemoglobin, serum alanine aminotransferase, serum aspartate aminotransferase and total serum bilirubin levels, were measured 24 h after treatment. The patients were discharged from the hospital 48 h after the treatment, if there were no any complications.

Table 2 Characteristics of HCCs in 56 cirrhotic patients treated with RFA

| Parameters | Numbers |
|-------------------|---------|
| Number of HCCs | 63 |
| One HCC | 50 |
| Two HCCs | 5 |
| Three HCCs | 1 |
| Type of HCCs | |
| Nodular | 63 |
| Infiltrating | 0 |
| HCC diameter (cm) | |
| > 1 < 2 | 19 |
| > 2 < 3 | 27 |
| > 3 < 4 | 12 |
| > 4 < 5 | 5 |
| Mean | 2.8 |

Table 3 Clinical findings in 23 cirrhotic patients with HCC recurrence after RFA

| Parameters | Numbers |
|----------------------------------|-----------------------|
| Total patients | 23 |
| Time of recurrence (mo) | |
| Range | 3-30 |
| Mean | 13 |
| Type of recurrence, <i>n</i> (%) | |
| Local unifocal | 2 (8.6%) |
| Different segments unifocal | 5 (21.8%) |
| Different segments multifocal | 16 (69.6%) |
| Treatment of recurrences | |
| RFA | 7 (unifocal) |
| TACE | 5 (multifocal) |
| Supportive treatment | 11 (multifocal) |
| Results of treatment | |
| RFA | Complete necrosis 7/7 |
| TACE | Complete necrosis 1/5 |

Assessment of therapeutic efficacy

To evaluate the response to RF therapy, contrast-enhanced CT with the same parameters as the pre-treatment scanning was performed 1 mo after the procedure; tumor necrosis was considered complete when no foci of enhancement were seen within the tumor or at its periphery on CT scans. Safety margin surrounding the ablated tumor was obtained in all the cases where tumor necrosis was considered complete. Every three months, the patients subsequently underwent color power Doppler ultrasound associated with AFP dosage; and in the event of modifications of the ultrasound picture or a progressive increase in AFP value, a CT scan was performed.

RESULTS

A total of 63 HCCs were treated with RFA in the 56 patients enrolled in the study. Two HCCs, measuring 3.5 and 4 cm in diameter, were non-responders to the treatment with RFA with persistence of the vascular signal after two sessions together with an increase in volume in the second (4-4.7 cm in maximum diameter). They were therefore subjected to chemoembolization with a positive result for the first but a progression of the disease in the second despite the additional therapy. The overall percentage of complete necrosis was 96.8% (61/63 of the lesions treated). In 54 HCCs, corresponding to 85.5% of the total, complete necrosis was achieved with just one session with a single insertion of the needle, while a second session was carried out in the remaining 7 HCCs after the first control CT scan due to persistence of vascularized areas of the tumor, again with a single insertion; a total of 68 treatments were necessary to achieve complete necrosis of the 61 responder HCCs. The diameters of the HCCs that required two sessions for complete necrosis were 3 cm in one case, 4 cm in one case and larger than 4 cm in the remaining 5 cases. The RF application time ranged from 2.5 to 15 min for each electrode insertion; the mean RF time was 10.4 min per session. All patients had an increase of echogenicity in the ablated region during RFA, suggesting generation of microbubbles in the ablated hepatic tissue.

As regards complications, 34 (60.7%) patients reported pain requiring the administration of analgesics during the treatment, while practically all subjects presented a moderate and transitory increase in transaminases at the 24 h hematological checks with no other laboratory test alterations. Major complications occurred in 3 of 72 cases (4%), including right pleural effusion in 1 case, cholecystitis in 1 case and liver failure in 1 case.

The post-treatment follow-up varied from 8 to 56 mo with a mean of 32.3 mo; during the follow-up, we detected 23 cases of recurrence (41%), with onset varying from 3 to 30 months after treatment and a mean of 13 mo. The recurrence was unifocal in 7 cases and multifocal in 16 cases; two of the unifocal cases were local recurrences, while the remaining five were localized in another segment. Of the 23 cases of recurrence, the previously treated HCC was a single lesion in 22 cases and two lesions in 1 case; there was no relationship with the dimensions of the primary HCC which varied from 1.5 cm to 4.5 cm. Of the 22 previously treated single lesions, recurrence was unifocal in 7 cases and multifocal in the remaining 15 cases. In the unifocal recurrences, we carried out a new cycle of RFA therapy. Of the 16 multifocal cases, chemoembolization was performed in 5 cases, while treatment was limited to support therapy in view of the tumor diffusion and the general clinical conditions in the remaining 11 cases. The seven patients who underwent a new cycle of RFA therapy had positive results and were disease-free with a follow-up varying from 4 to 15 mo (mean 9 mo); four of the five patients treated with chemoembolization died due to progression of the HCC, while the other patient had a stable tumor situation 5 months after the treatment (Table 3).

Finally, 32 (57.1%) patients died during the follow-up, 15 from HCC, 11 from liver failure, 3 from esophageal varices bleeding, 1 from head stroke, 1 from cerebral hemorrhage and 1 from esophageal carcinoma. Among the patients who died from liver failure, 9 were Child-Pugh B and 2 were with Child-Pugh C (Table 4). Twenty-one patients were alive and disease-free at the end of the study.

Table 4 Clinical findings in 32 deceased cirrhotic patients treated with RFA for HCC

| Causes of death | Number of patients | Child-Pugh status | | |
|-----------------------------|--------------------|-------------------|----|---|
| | | A | B | C |
| HCC recurrence | 15 | 4 | 12 | |
| Liver failure | 11 | | 9 | 2 |
| Esophageal varices bleeding | 3 | | 3 | |
| Head stroke | 1 | | 1 | |
| Brain hemorrhage | 1 | 1 | | |
| Esophageal cancer | 1 | 1 | | |

DISCUSSION

The limitations to the surgical treatment of HCC due to site, age and associated clinical conditions have led to the development of alternative locoregional therapies of which PEI is the first and most widely used example. RF was subsequently introduced by Rossi *et al*^[8] in the attempt to overcome some of the limits of PEI, such as the need for multiple sessions, the difficulty in foreseeing the diffusion of the ethanol, and is now being used more and more as the first choice technique in the locoregional treatment of HCC^[13]. Also, in our department, RFA has gradually replaced PEI which we had been using since the mid 80 s and which is now reserved for selected cases. Our experience in RFA with the LeVein needle electrode has given us satisfactory results in terms of therapeutic efficacy, achieving complete necrosis in 85.5% of the lesions with just a single treatment, increasing to 96.8% with a second session, which are in agreement with the data reported in the literature^[10,14,15]. Necrosis was not achieved with RFA in two cases of HCC, as shown by the post-treatment CT scan, and we were unable to identify the reason for the lack of response with certainty; the diameters were 3.5 and 4 cm and the lesions were not close to large vessels whose blood flow could have dispersed the heat^[16]. Both were localized immediately under the hepatic dome and we therefore think it probable that the site interfered with the correct positioning of the electrode, reducing the efficacy of the treatment as previously reported^[15]. Among the 7 HCCs which required a second session, 6 cases had a diameter of 4-5 cm, while only in one case had the diameter of 3 cm; therefore, if we consider only the HCCs up to 3 cm in diameter, the percentage of complete necrosis with just a single treatment would rise to 97.8%. Our experience also confirms that diameter < 3 cm is the ideal condition for locoregional treatment with RFA as with PEI^[13,14,17]. However, in lesions measuring 3-5 cm, RFA with a LeVein needle gave acceptable results with complete necrosis at the first treatment in 64.7% of cases, the remaining 35.3% required a second session for complete necrosis. As previously reported by Livraghi *et al*^[10], the lesser number of treatment sessions with respect to PEI makes it possible to reduce the time employed by the hospital staff and to considerably shorten the duration of the treatment and eliminate the stress of repeated sessions in most cases. One factor that can increase the volume of the area of necrosis and reduce the number of necessary treatment sessions is represented by

what Livraghi *et al*^[10] defined as the “oven effect” caused by the fact that cirrhotic liver surrounding individual HCC nodules acts as a thermal insulator that increases tissue heating during RF therapy, making it possible to treat non-infiltrating tumors in a single treatment session in the majority of the cases.

The LeVein needle electrode we used in this study has ten expandable inner hooks in a single outer sheath to obtain even greater flux of the radio wave in the ablated tissue. Through this, the needle produces a greater spherical area of thermal ablation than can be obtained by the previous four expandable hooks needle, assuring a safety margin if the tumor is within the range of ablation. Furthermore, the LeVein needle can hold itself securely within the area of ablation by expanding the hooks; this may help in the treatment of tumors in areas susceptible to the respiratory movement of tissue. The LeVein needle has some limitations, the first represented by the 15 gauge caliber and a greater risk of bleeding can be expected; however, we did not experience any hemorrhagic complications, and the literature also has not reported a greater incidence of hemorrhagic complications with respect to smaller gauge needles, such as 18 gauge cool-tip needle^[11]. An 18-gauge LeVein needle with 2.5 cm and 3 cm maximum deployment diameter of the hooks has recently become available, eliminating the difference in gauge; in the two cases treated with this finer needle, we did not encounter problems compared to the traditional one. A second limitation is the low visibility of the tips of the hooks under ultrasonography which sometimes makes it difficult to assess the correct location of the hooks with possible unexpected complications^[15].

In our experience, not only was RFA with the LeVein needle effective, it also proved safe with a low incidence (4%) of major complications, similar to the percentage reported in the literature^[3,11,18,19,20]. In two cases (pleural effusion and cholecystitis), the complication was because the HCC was close, respectively, to the diaphragm and the gall bladder with consequent thermal irritation of the pleura and of the gall bladder wall. In spite of being quickly resolved with medical therapy and without sequelae, this confirms the need to take particular care in treating lesions in the sites at risk as previously described^[21]. In the third case, functional liver failure occurred with ascites after treatment of a 2.3-cm HCC of liver segment III in a Child-Pugh class C patient. The HCC had been subjected to a single session of RFA lasting 7.8 min. Medical therapy achieved a partial improvement of the clinical conditions; however, the patient subsequently died 4.5 months after treatment from liver failure without HCC recurrence. It should be further stressed that in patients with HCC on Child-Pugh class C cirrhosis locoregional treatments should not be carried out due to the greater risks and the advanced stage of the cirrhosis which *per se* involves a poor prognosis for survival^[22]. The exception to this rule is treatment of patients on the waiting list for orthotopic liver transplant (OLT) when there is a long waiting time, as is frequently the case in almost all the transplant units in Italy^[23,24]. Two of the three Child-Pugh class C patients that we treated, including the one in whom the complication occurred, were on the waiting list for OLT. The second

patient on the waiting list underwent OLT 15 months ago and is alive and free of recurrence.

We did not find any cases of post-RFA tumor seeding. In contrast, a study by Spanish authors has reported a high incidence of post-RFA seeding^[25] which has not, however, been confirmed in any of the other studies reported in the literature. A recent study has demonstrated an incidence of post-RFA seeding even lower than that of post-PEI, both with expandable needles and with cooled needles^[11].

As far as complications are concerned, a recent Italian study showed that despite achievement of complete local necrosis, rapid intrahepatic neoplastic progression was observed in four patients (4.5%) after treatment. The authors experienced that high AFP levels and location of the tumor near the portal vein branches were associated with this complication^[26]. Moreover, 75% (3/4) patients had a poor differentiation of the tumor^[26]. This is the only report of this type in the literature to date and, while attention should be paid to this possibility, further studies are necessary for its confirmation. However, in our series, we have not found any similar occurrences to date.

Evaluation of the tissue necrosis is fundamentally important in order to decide whether further treatment is necessary and is currently mainly based on imaging techniques to assess the persistence of lesion vascularization, rather than on a biopsy which can not give a complete sampling of the lesion. The gold standard technique is certainly the contrast-enhanced CT scan, and we, based our post-treatment evaluation on this technique, performed it in all patients after thirty days. This is the most common interval as it allows any remaining vital tissue to be detected within a period of time that is short enough to prevent significant progression of the disease and to not burden an already overloaded radiology service with an excessive number of CT scans. It also prevents the risk of interpreting the hyperdense rim present in the majority of cases on CT scans obtained in a earlier period after RFA. This hyperdense rim is the expression of the early parenchymal inflammatory rim around the treated tumor as proved in resected specimens^[27]. In our series, the HCCs with signs of activity at the one-month CT scan underwent a second session of RFA with complete necrosis in 100% of cases; the time lapse did not have any negative effect on the result of the treatment. The first data on the use of contrast-enhanced ultrasound (CEUS) with second generation contrast medium have recently been reported and are extremely encouraging, particularly as regards early 24 h evaluation of post-RFA persistence of activity^[28-30]. The possibility to obtain by CEUS data comparable with the ones obtained by CT would make it possible to use a more rapid method and, if necessary, to carry out a second treatment during the same session, thus further reducing the duration of the therapeutic cycle with advantages for both patients and hospital staff.

In our series, HCC recurrence occurred in 41% (23/56) patients, a lower percentage than those reported in the literature^[31]. This may be due to the lower number of patients and to the shorter follow-up compared to other studies. The percentage of local recurrence was only 3.1% (2 patients), in line with what was reported by other authors^[31], while recurrence occurred in other liver

segments with respect to the initial one in the remaining 21 patients, thereby confirming the validity of RFA in achieving complete necrosis of the treated HCCs. In both cases of local recurrence, the lesion diameter was > 3 cm (4 cm and 4.5 cm), confirming the limits of locoregional therapy in larger tumors. One factor that may contribute in determining recurrence is the presence of satellite nodules that can not be identified by imaging^[32]. Livraghi *et al*^[13] supposed that the "oven effect" may partially explain the limited success in treating satellite lesions. They believe that peritumoral fibrotic tissue that is interposed between the main tumor and satellite lesions may limit heat diffusion from the tumor center to the satellites. The association of RFA and transcatheter arterial chemoembolization (TACE) has been proposed as a means of reducing the number of local recurrences and the initial results seem encouraging^[33,34]. The problem is different for recurrence in other sites, which remains high regardless of the type of treatment used, with values ranging from 60% to 80% after four years follow-up in series reported by Japanese authors^[31].

In our experience, it was possible to treat all 7 patients with unifocal recurrence with RFA, achieving complete necrosis of the lesion in all cases, but only 5 of the 16 multifocal recurrences with TACE and with a positive result in only one case. It is becoming important in the clinical setting to establish treatment methods to prevent recurrences at other sites, such as the use of interferon or anti-inflammatory therapy particularly to decrease the incidence of multicentric recurrences^[35-38]. Therefore, even after achieving the local curative ablation by RFA, it is important to closely follow-up the patients for early detection and treatment of recurrence.

The rather limited number of patients and the too short mean follow-up period in our study do not allow us to draw conclusions on the efficacy of RFA of HCCs in improving survival. Nevertheless, by analyzing the causes of death in the patients who died during follow-up, it can be seen that while none of the Child-Pugh class A patients died from cirrhosis-linked causes, 9 patients in class B and 2 out of 3 in class C died as a result of progression of the cirrhosis, and the third patient in class C is alive after OLT. In addition, another 3 patients in class B died as a result of bleeding from esophageal varices. This further confirmed the importance of the stage of cirrhosis in patient survival, so as to exclude class C patients from any ablation treatment except for those on the waiting list for OLT. In our series, however, 46.8% (15/32) patients died as a direct result of the HCC; our data also indicated that, as mentioned earlier, it is extremely important to improve the prognosis of patients who have undergone local ablation therapy by treating the background liver disease, using chemopreventive drugs, such as interferon. Regarding the previous data on the long-term survival of patients treated with RFA for HCC, it should be pointed out that although the history of RFA is not long enough for analysis, when analysis was limited to cases of primary liver cancer in which local curative therapy was achieved, the 5-year survival rate was relatively high (66-82%), comparable to that of resection^[17,31,39-41].

In conclusion, percutaneous RFA by means of the

LeVeen needle electrode appears to be an effective, safe and relatively simple procedure for the treatment of HCC lesions in patients with cirrhosis, obtaining the best results in HCCs < 3 cm in diameter. We currently prefer to use RFA rather than PEI because RFA is capable of achieving complete tumor necrosis in fewer treatment sessions compared with multi-session PEI, reserving PEI for HCCs that are difficult to approach or located in areas where RFA is considered unsafe, such as HCC adjacent to large vessels, the main bile duct or the gall bladder. Despite the good results as regards the necrosis of the HCC lesions, it is not yet possible to define the effect of RFA on the long-term survival of treated patients, even though the initial data are encouraging. Notwithstanding the importance of the stage of cirrhosis for survival, HCC recurrence after RFA is a significant cause of death in these patients. On the basis of the recent data reported in the literature, it is probable that in the future the prognosis can be improved by the use of chemopreventive drugs, such as interferon for the prevention of recurrence after curative local treatment.

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Flow cytometry assay of myeloid dendritic cells (mDCs) in peripheral blood during acute hepatitis C: Possible pathogenetic mechanisms

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Abstract

AIM: To assess the expression of myeloid dendritic cells (CD11c+) subset during acute HCV hepatitis and its possible involvement in natural history of the infection.

METHODS: We enrolled 11 patients with acute hepatitis C (AHC) (Group A), 10 patients with acute hepatitis A (AHA) (as infective control-Group B) and 10 healthy donors (group C) in this study. All patients underwent selective flow cytometry gating strategies to assess the peripheral number of the myeloid dendritic cells (mDCs) to understand the possible role and differences during acute hepatitis.

RESULTS: Eight of 11 patients with acute HCV hepatitis did not show any increase of mDCs compared to healthy individuals, while a significant decrease of mDCs was found in absolute cell count ($z = -2.37$; $P < 0.05$) and percentage ($z = -2.30$; $P < 0.05$) as compared with AHA. On the contrary, the remaining three patients of the group A had a higher mDCs number and percentage as occur in group B. Interestingly, after six months, those patients did not show any increase of mDCs subset were chronically infected, while the three subjects with an increase of peripheral mDCs, as in HAV acute infection, resolved the illness.

CONCLUSION: The lack of increase of mDCs during acute hepatitis C might be an important factor involved in chronicization of the infection.

INTRODUCTION

Hepatitis C virus (HCV) infection represents one of the most important causes of chronic viral hepatitis, liver disease, and hepatocellular carcinoma^[1]. Many mechanisms have been proposed to account for the ability of HCV to elude the host defense and progress towards chronic disease: the high rate of the mutation as well as the ability of the virus to influence T helper cell subsets during the acute infection. Indeed, an early Th2 predominant immune response seems to influence the natural history of the infection^[2,3]. Even if, several research studies have focused attention on the T lymphocytes (CD4+ and CD8+) activity in acute and chronic hepatitis C^[4,5], however, the frequency of the cells involved in the presentation of the antigen (APCs) and so in the immune response activation is not fully elucidated. Among these subsets, the dendritic cells (DCs) have one of the most important roles in the host defense, but few data are available on these cells during the acute hepatitis C. That subset, divided in plasmacytoid DCs (pDCs) and myeloid DCs (mDCs), expresses high levels of MHC class I and II antigens, as well as costimulatory molecules, playing an essential role in triggering primary immune response^[6]. Particularly, the mDCs are involved in Th1 network activation^[7], that is fundamental to clear HCV infection^[2,3]. The aim of the present study was to use flow cytometry as a tool to enumerate mature mDCs in peripheral blood of patients with acute HCV infection and consequently to assess the ability to present the antigen and activate the immune response against the virus as well as its possible influence on disease progression. Thus, we

presented, for the first time to our knowledge, a study on the frequency of peripheral myeloid dendritic cells (mDCs) (CD11c+) in two hepatitides due to RNA hepatotropic virus characterized by a common natural immune system pathway^[8-10]: one HCV which frequently leads to chronicity; and the other HAV which normally leads to a self-limited infection with a complete resolution, thus as control population, we investigated healthy individuals.

MATERIALS AND METHODS

Patients

Following the approval of our Local Ethical Committee, the study was carried out on 31 subjects, who were divided into three groups. Group A: 11 patients with acute C hepatitis; Group B: 10 patients with acute A hepatitis; Group C: 10 normal individuals as controls. All patients had been admitted to our Infectious Disease Emergency Unit during the symptomatic phase, at least after one week from the first symptoms. Regarding to the group A, the criteria for diagnosis of acute hepatitis C were: (1) at admittance, HCV-RNA positive and anti-HCV negative or documented HCV-RNA negativity before admission otherwise seroconversion from anti-HCV negative to positive ELISA test; (2) negative clinical history of autoimmune disease, chronic viral hepatitis or other viral infections, including HIV; (3) serum alanine aminotransferase (ALT) levels at least 10 × upper limit of normal value (ULN < 40 IU/mL); and (4) absence of alcohol or drug abuse. Of note that our hospital is a reference center for infectious disease, moreover, most of our patients were ex-prisoner and so they underwent to periodical control.

All HAV-infected patients were negative for other chronic viral hepatitis (HBV and HCV), alcohol/drug abuse, other associated autoimmune disorders or infectious disease.

Since none of patients of group A underwent IFN treatment, they were evaluated at time 0 (T0) and after at least six months Time 6 (T6) following their laboratory parameters to asses a possible progression towards chronic infection (positive HCV-RNA qualitative assay and hypertransaminasis) or its resolution, as well as their mDCs frequencies.

Blood samples were collected from all patients on admittance to our unit, at the beginning of clinical symptoms (T0). All biochemical and viral assays were performed at our centralized laboratory. The HCV-RNA qualitative and quantitative assays were performed using the Amplicor Roche system with the cut-off values set to 50 and 600 UI/mL, respectively.

The mDCs assessment was executed at our immunological laboratory by a three-color flow cytometry using a Beckman Coulter Profile system. The mDCs assay involved the use of anti-ILT3, anti-CD14 and anti-CD11c monoclonal antibodies (mAb) labelled with the fluorochromes -PC5,-FITC,-PE, applying a particular gate strategy, according to the criteria of Beckman Coulter. All monoclonal antibodies were applied following the manufacturer's instructions and on the basis of our previous experiences. Briefly, after the cells were gated using a side scatter profile to exclude granulocyte and possible debris,

the cells were further gated using CD14+ mAb marker to select a population of monocytes and pro-myeloid dendritic cells, such marker is not expressed on plasmacytoid cells and so its usage gives us the ability to early select the mDCs^[11]. In addition, the cells were further gated using ILT3-mAb to obtain purified mDCs population^[12]. Finally, a third gate was applied using CD11c to obtain the only mature mDCs (CD11c+)^[13] (Figure 1). Coupled to this cytometry analysis, we also evaluated the total CD4+ T cells to asses the possible correlation between T lymphocytes and mDCs.

Statistical analysis

Considering the small number of patients, we used non-parametric test. Spearman test was applied particularly to asses possible correlation, and on the other hand, *U* Mann-Whitney test (two tailed) was performed to evaluate difference among the groups. The results were considered statistical significant at $P < 0.05$.

RESULTS

Table 1 shows the descriptive analysis of the standard laboratory, virological and immunological parameters. In group A, nine patients were genotype 1b, while the other two had genotype 2a/2c. The mean value of mDCs in the normal donors was 1350 cells, representing about the 15% of the ILT3 cells. Eight of 11 patients with acute hepatitis C showed to have a lower absolute cell count percentage of peripheral blood mDCs when compared to acute hepatitis A (Mann-Whitney *U* test; $z = -2.37$, $P < 0.05$ and $z = -2.30$, $P < 0.05$) (Figure 2), while they did not display any statistical significant differences with respect to group C (healthy individuals), thereby suggesting the lack of enrollment in peripheral blood of this dendritic cell subset during the acute infection. The remaining 3 patients (3/11; two with genotype 2a/2c and one genotype 1) showed an increase of mDCs presenting an absolute cell count and percentage analogous to patients with acute hepatitis A (Table 1). After six months, patients who at T0 showed an absolute number and percentage of mDCs like healthy individuals had positive HCV-RNA qualitative assay, hypertransaminases (2 time upper limit normal value, 92 IU/mL) and also preserved the same frequency of mDCs (Table 1). Of note that 3/11 of patients who showed an increase of mDCs were found to be negative for hypertransaminasemia and HCV-RNA after 9 months when they came back our department. Finally, a strong positive correlation (Spearman's test; $r = 0.90$, $P < 0.05$) between the mDCs and CD4+ was found in group B, while it was not demonstrated in group A. Noteworthy, no correlation was pointed out among HCV-RNA, transaminases and the mDCs both at T0 and T6, suggesting the lack of a direct involvement in liver necrosis of viral cycle.

DISCUSSION

mDCs have an important role during the infection^[6,14], therefore, one of the most vital strategies of the virus could be to inhibit or interfere with their function. Indeed,

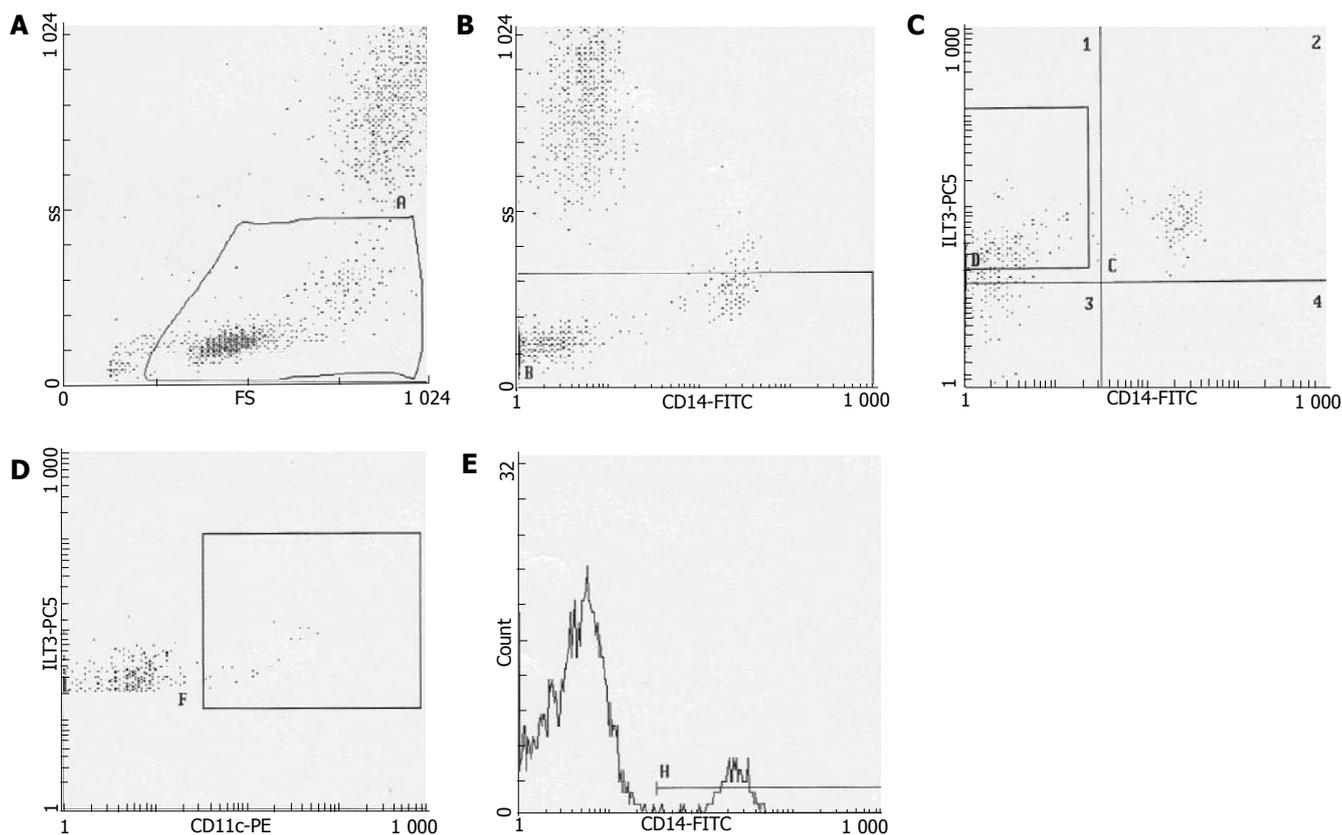


Figure 1 Gate strategies for the mDCs CD11c+ evaluation. The letter's order represents the sequential gates used to measure the frequency of mDCs. (A) Side scatter vs forward scatter gate analysis; (B) CD14+ cells analysis vs side scatter to select CD14+ cells avoiding debris; (C) characterization of the cells having high expression of ILT3, as marker of purified myeloid dendritic lineage, on CD14+ subset; (D) evaluation of cells expressing CD11c+, as marker of mature myeloid dendritic cells, on the whole ILT3 gated cells to obtain the mDCs; and (E) histogram of CD14-FITC mAb fluorescence.

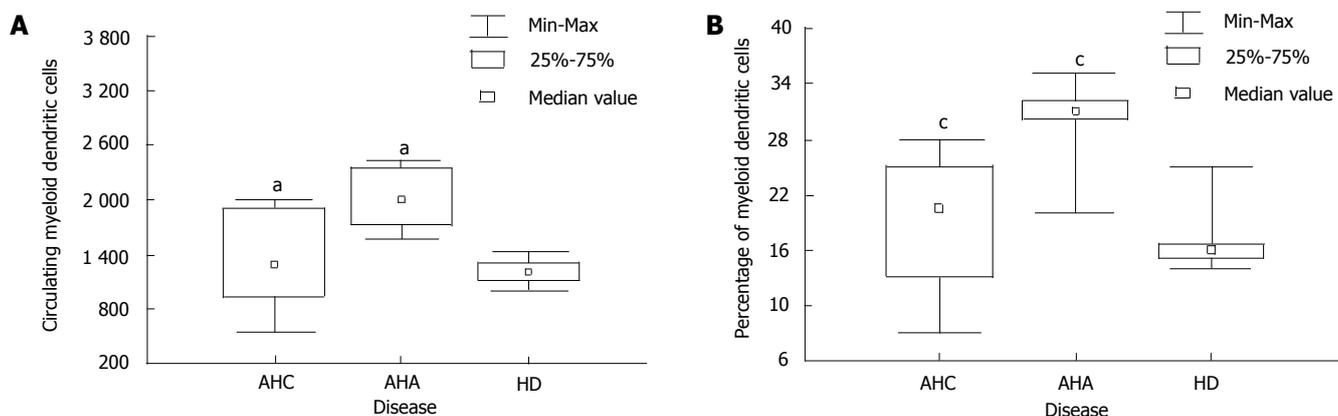


Figure 2 Differences in absolute cell count and percentage of mDCs among the examined groups. The patients having HAV acute infection (AHA) displayed an increase of mDCs as compared to the subjects with acute hepatitis C (AHC) who displayed to have the same frequency as the healthy donor (HD). : Significant differences in absolute cell count ($z = -2.37$; $^aP < 0.05$) and percentage ($z = -2.30$; $^cP < 0.05$) (Mann Whitney *U* test).

some viruses can evolve to present strategies to diminish the circulating dendritic cells evading the immune surveillance. Previous studies reported a decrease of DCs during different viral infectious disease and most recently in chronic hepatitis C^[14,15]. Particularly during chronic HCV infection, it has also been shown that the virus is able to slow down the DCs by direct infection^[15]. Nevertheless, it is unknown if the virus has influence on the circulating DCs during acute infection too. Although the present

investigation was carried out on a small group of patients, however, this simple gate strategy in flow cytometry is probably the first to show that patients who demonstrated to have the same absolute number and percentage of mDCs as healthy individuals evolved towards a chronic disease, while those having an increase of mDCs, such as in HAV, were not chronically infected. Even if the number of patients resolving the infection is too small to be suitable for any statistical test, it is clear considering the

Table 1 Laboratory and immunological parameters of the enrolled patients (mean \pm SD, n, %)

| Parameters | A-T0 | A-T6 | A-res | B | C |
|--------------------------------|---------------------------|-----------------|---------------------------|-----------------|-----------------|
| Age (yr, mean \pm SD) | 42 \pm 2 | 42 \pm 3 | 46 \pm 5 | 35 \pm 3 | 32 \pm 5 |
| Gender | | | | | |
| Male (n=9) | 8/11 | 8/11 | 1/11 | 9/10 | 6/10 |
| Female (n=2) | 0/11 | 0/11 | 2/11 | 1/10 | 4/10 |
| ALT level (<40 U/L) | 1 325 \pm 360 | 92 \pm 12 | 1 620 \pm 220 | 1 328 \pm 325 | <40 |
| AST level (<40 U/L) | 817 \pm 435 | 84 \pm 9 | 942 \pm 160 | 996 \pm 73 | <40 |
| HCV-RNA (IU/mL, mean \pm SD) | 239 \pm 2 $\times 10^3$ | + | 198 \pm 3 $\times 10^3$ | - | - |
| HBsAg | - | - | - | - | - |
| Genotype | | | | | |
| 1 | 8/11 | 8/11 | 1/11 | n.a. | n.a. |
| 2a/2c | 0/11 | 0/11 | 2/11 | n.a. | n.a. |
| CD4+ | 993 \pm 333 | 850 \pm 240 | 850 \pm 240 | 558 \pm 167 | 922 \pm 345 |
| CD11c+% | 19 \pm 9% | 17 \pm 7% | 26 \pm 8% | 30 \pm 5% | 15 \pm 4 % |
| CD11c+ cells | 1 220 \pm 558 | 1 312 \pm 345 | 1 760 \pm 232 | 1 980 \pm 311 | 1 350 \pm 200 |
| Mode of infection | | | | | |
| Sexual transmission | 1/11 | | | 0 | |
| Unclear | 8/11 | | | 0 | |
| Surgical procedures | 2/11 | | | 0 | |
| Alimentary | 0 | | | 10/10 | |

A-T0 and A-T6 represent the data of the patients who were chronically infected after six months. T0 = at the admission; T6 = after six months; A-res= data at Time 0 of the patients who were negative for HCV-RNA and hypertransaminasemia after six months; n.a. = not applicable.

flow cytometry data that this subset has a possible connection to the pathogenesis of a persistent infection. In this regard, the results relatively to the increase of mDCs during a self-limited RNA viral hepatitis like HAV, seem to confirm the hypothesis of a role for those peripheral blood professional APCs subset in the resolution of the viral infection, probably enrolling CD4+ T cells as the correlation suggested. The mechanisms hidden behind these events, through which HCV could participate in decreasing mDCs in peripheral blood, are still unclear. Nevertheless, it has recently been shown the ability of HCV to interfere with TLR 3 activity during the immune system response^[16], since it has an important role in mDCs life cycle and function^[6,9,10,17]. Thus, the lack of expansion occurring in patients during acute HCV infection by different ways could represent an important additional skill of the virus to elude the first line of host defense represented by the innate immunity. Although our study did not assess the direct viral infection of DCs, it clearly documents that HCV is able to evolve a kind of strategy to boost its survival by preventing the increase of mDCs during the acute infection in patients evolving in chronic hepatitis. Of note that mDCs are able to activate a Th1 immune network, and that it is well known to be necessary to clear HCV^[4,5,18], so their inactivation might have a great influence on host defense during acute infection.

In conclusion, we speculate that during acute hepatitis C, the virus might prevent the activation of circulating myeloid dendritic cells (ILT3/CD11c+) conditioning the natural history of the hepatitis. Thus, an early assessment by means of flow cytometry of mDCs using our gate strategy could give useful suggestions about the possible progression of infection, thereby proposing the early enrolment of the patients in IFN-treatment, particularly for the genotype 1. Supplementary studies, using flow cytometry assay

coupled to functional analysis as well as assaying the possible variation of mDCs peripheral blood expression after interferon plus ribavirin treatment, should be carried out on a wider population. In fact, this subset could be a possible target for an immunotherapy to ameliorate the antigen presenting function and so the immune response

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RAPID COMMUNICATION

Inhibitory effect of *Patrinia scabiosaefolia* on acute pancreatitis

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Abstract

AIM: To investigate the effect of *Patrinia scabiosaefolia* (PS) on the cholecystokinin (CCK) octapeptide-induced acute pancreatitis (AP) in rats.

METHODS: Wistar rats weighing 240-260 g were divided into three groups: (1) Normal saline-treated group; (2) treatment with PS at 100 mg/kg group, in which PS was administered orally, followed by subcutaneous administration of 75 µg/kg CCK octapeptide three times after 1, 3 and 5 h, and this whole procedure was repeated for 5 d; (3) treatment with saline group, in which the protocols were the same as in treatment group with PS. We determined the pancreatic weight/body weight ratio, the levels of pancreatic HSP60, HSP72 and the secretion of pro-inflammatory cytokines. Repeated CCK octapeptide treatment resulted in the typical laboratory findings of experimentally induced pancreatitis.

RESULTS: PS reduced the pancreatic weight/body

weight ratio, the levels of serum amylase and lipase, and inhibited expressions of pro-inflammatory cytokines in the CCK octapeptide-induced AP. Furthermore, PS pretreatment increased the pancreatic levels of HSP60 and HSP72.

CONCLUSION: Pretreatment with PS has an anti-inflammatory effect on CCK octapeptide-induced AP.

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Key words: Acute pancreatitis; *Patrinia scabiosaefolia*; CCK octapeptide

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INTRODUCTION

Acute pancreatitis (AP) is characterized by local pancreatic inflammation as well as a systemic inflammatory response^[1,2]. Histologically, AP is characterized by interstitial edema, vacuolization, inflammation, and acinar cell necrosis^[3,4]. A widely used model to study these responses is acute pancreatitis induced in rats and mice by high doses of cholecystokinin (CCK) or its analogue, cerulein^[5]. CCK is a major physiologic regulator of digestive enzyme secretion by the pancreatic acinar cell; however, supraphysiologic doses of CCK (in excess of those that stimulate maximal secretion of digestive enzymes) are injurious to the pancreas, causing pancreatitis that mimics many features of the human disease^[5-8].

Cells could respond to heat shock or other stress with the rapid synthesis of heat-shock proteins (HSPs). The induction of the heat shock response enhances the ability of the cells to overcome the effects of the stress^[9]. These proteins are classified by their molecular mass (e.g., HSP60 and HSP72). It was reported that the pre-induction of HSP expression has a protective effect against cerulein-induced pancreatitis in rats or choline-deficient methionine-supplemented diet model pancreatitis in mice^[10-12].

Besides, with increasing neutrophil migration to the pancreas, a variety of inflammatory cytokines are released.

Pro-inflammatory mediators, such as tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β) and IL-6, have been related to inflammatory changes in AP. There are considerable evidences that pro-inflammatory cytokines play a central role in AP and may mediate the systemic complications of AP^[13,14].

Iridoid compounds represent a large group of the cyclopentano[d]pyran monoterpenoids found in many plants. These compounds exhibit various antimicrobial, antitumoral, hemodynamic, and anti-inflammatory activities^[15]. *Patrinia scabiosaefolia* (PS) (Valerianaceae) is well-known in oriental traditional medicine. Its roots or whole plants have been used for treatment of various diseases, including edema, appendicitis, endometritis, and inflammation^[16]. However, the effect of PS on AP has not been investigated as yet. Hence, in this study, we aimed to investigate the effect of PS on the CCK octapeptide-induced AP. We examined pancreatic weight/body weight ratio, serum amylase and lipase levels, and also investigated the effect of PS on pancreatic HSP60 and HSP72 expression in CCK octapeptide-induced AP. We wished to evaluate whether PS can block pro-inflammatory cytokine synthesis during CCK octapeptide-induced AP.

MATERIALS AND METHODS

Materials

Avidin-peroxidase and 2'-AZINO-bis (3-ethylbenzothiazoline-6-sulfonic acid) tablets substrate and CCK-8 were purchased from Sigma (St. Louis, MO, USA). Anti-HSP60 and anti-HSP72 antibodies were purchased from Stressgen (British Columbia, Canada). Anti-rat TNF- α , anti-rat IL-1 β and anti-rat IL-6 antibodies were purchased from R&D Systems (Minneapolis, MN, USA).

Preparation of PS

PS was prepared by decocting the dried prescription of herbs with boiling distilled water. The decoction time was about 3 h. Their voucher specimens were deposited at the Herbarium at the College of Oriental Medicine, Kyung-Hee University.

Animal models

Male Wistar rats weighing 240-260 g were kept at a constant room temperature of 25 °C with a 12-h light-dark cycle, and were allowed free access to water and standard laboratory chow. The rats were fasted for 16 h before the induction of AP. Rats were divided into three groups, each group containing 6-8 rats. The experiments performed in this study were approved by the Animal Care Committee of the University.

In vivo models of pancreatitis

In PS-treated group, 100 mg/kg PS was systemically administered orally, followed by subcutaneous administration of 75 μ g/kg CCK octapeptide three times after 1, 3, and 5 h, and this whole procedure was repeated for 5 d. In AP group, saline was systemically administered orally instead of PS, but otherwise the protocol was the same as in treatment group with PS. Normal untreated

group received physiological saline orally instead of PS. The animals were sacrificed by exsanguinations through the abdominal aorta 12 h after the last CCK octapeptide injection. The pancreas was quickly removed, cleaned from fat and lymph nodes, weighed, and frozen at -70 °C until use. Rats were treated in accordance with the current law and the NIH Guide for Care and Use of Laboratory Animals.

Pancreatic weight/body weight ratio

This ratio was utilized to evaluate the degree of pancreatic edema.

Western blotting

Western blot analysis of pancreatic HSP60 and HSP72 levels was performed from the cytosolic fraction of the pancreas homogenate. Thirty micrograms of protein was loaded per lane. Samples were electrophoresed on a 100 g/L SDS-PAGE according to the method of Laemmli^[17]. The gels were either stained with Coomassie brilliant blue (to demonstrate equal loading of proteins for Western blot analysis) or transferred to a nitrocellulose membrane for 2 h at 300 mA. Membranes were blocked in 50 g/L nonfat dry milk for 1 h and incubated with anti-HSP60 and anti-HSP72 antibodies. After washing in PBS/Tween-20 three times, the blot was incubated with secondary antibody for 30 min, and the antibody-specific proteins were visualized by the enhanced chemiluminescence detection system according to the recommended procedures (Amersham Corp. Newark, NJ).

Enzyme-linked immunosorbent assay (ELISA)

ELISA for TNF- α , IL-1 β and IL-6 was carried out in duplicate in 96-well plates (Nunc, Denmark) coated with each of 100 μ L aliquots of anti-rat TNF- α , anti-rat IL-1 β and anti-rat IL-6 monoclonal antibodies at 1.0 μ g/mL in PBS (pH 7.4), followed by incubation overnight at 4°C. The plates were washed with PBS containing 0.5 g/L Tween-20 (Sigma, St. Louis, MO, USA) and blocked with PBS containing 10 g/L BSA, 50 g/L sucrose and 0.5 g/L NaN₃ for 1 h. After additional washes, standards were added and incubated at 37 °C for 2 h, followed by washing of the wells, addition of each of 0.2 mg/L of biotinylated anti-rat TNF- α , anti-rat IL-1 β and anti-rat IL-6 antibodies, and incubation at 37 °C for 2 h. After washing the wells, avidin-peroxidase was added and plates were incubated for 20 min at 37 °C. Wells were again washed and ABTS substrate was added. Color development was measured at 405 nm using an automated microplate ELISA reader. A standard curve was run on each assay plate using recombinant TNF- α , IL-1 β and IL-6 in serial dilutions.

Measurement of serum amylase and lipase

Arterial blood samples for determinations of serum amylase and lipase were obtained 12 h after induction of pancreatitis. Rats were anesthetized with intraperitoneal injection of ketamine (80 mg/kg) and xylazine (4 mg/kg). After anesthetization, blood was withdrawn from heart of each rat into syringes. Serum amylase was measured by using an ADIVA 1650 (BAYER, USA). Serum lipase was

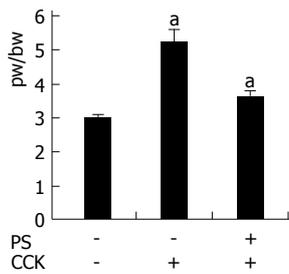


Figure 1 Effect of PS on pancreatic weight/body weight ratio (pw/bw) in CCK octapeptide-induced AP. Data are expressed as mean \pm SE for 6 animals per group. $P < 0.05$ vs control group.

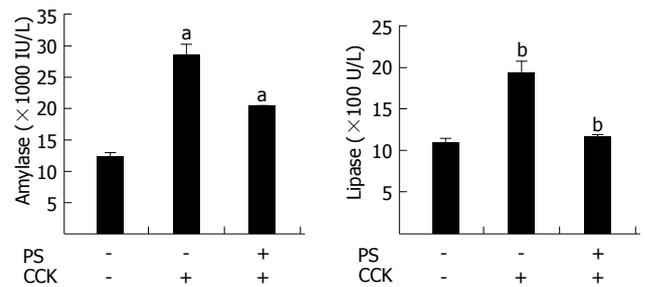


Figure 2 PS inhibition of serum amylase and lipase levels in CCK octapeptide-induced AP in rats. Values are expressed as mean \pm SE for at least 6 animals per group. $P < 0.05$ vs control group.

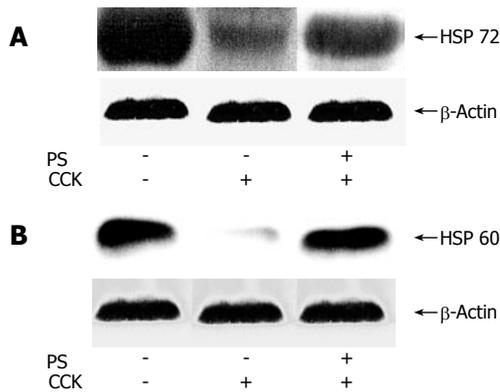


Figure 3 Western blot analysis of effect of PS on HSP60 and HSP72 expressions in CCK octapeptide-induced AP. **A:** HSP72; **B:** HSP60.

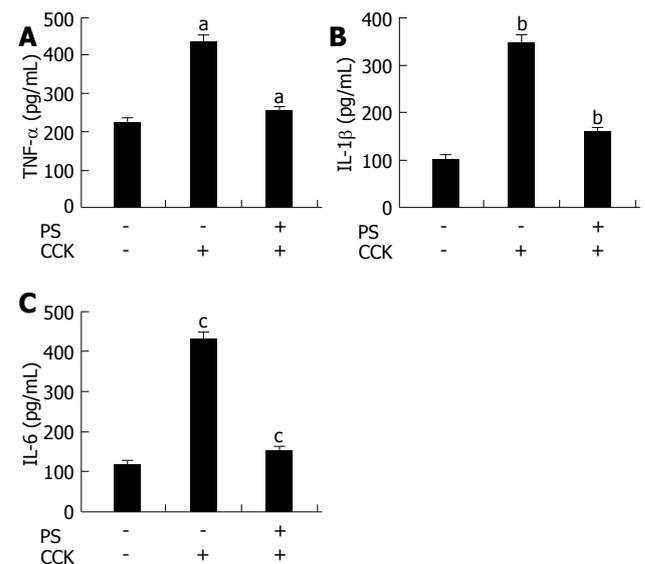


Figure 4 Effect of PS on TNF- α , IL-1 β and IL-6 secretion in CCK octapeptide-induced AP. Data are expressed as mean \pm SE for 6 animals in each group. $P < 0.05$ vs control group.

measured by using a Cobas-mira (Roche, USA).

Statistical analysis

Results were expressed as means \pm SE. The significance of changes was evaluated using student's *t* test. Differences between the experimental groups were evaluated by using analysis of variance. *P* values less than 0.05 were considered statistically significant.

RESULTS

Effect of PS on pancreatic weight/body weight ratio

It has been shown that pancreatic weight (pw)/body weight (bw) ratio is increased in CCK octapeptide-induced pancreatitis in rats^[18]. To assess the effect of PS on the pw/bw ratio during CCK octapeptide-induced AP, we divided the pancreatic weight by the body weight of rats. We found that PS significantly decreased pw/bw ratio compared with saline-treated group ($P < 0.05$) (Figure 1).

Pancreatic serum amylase activity and lipase activity

The levels of serum amylase and lipase are most commonly obtained as biochemical markers of pancreatic disease, particularly AP. Amylase activity in serum has been used for many years for the evaluation of patients with acute abdominal pain and suspected pancreatic disorders^[19]. Elevation of the levels of pancreatic lipase in plasma or serum is often considered to be the most sensitive and specific marker of AP^[20]. PS reduced the

activities of serum amylase and lipase in CCK octapeptide-induced AP (Figure 2).

Effect of PS on HSP60 and HSP72 expression in CCK octapeptide-induced AP

It was reported that the pre-induction of HSP expression has a protective effect against cerulein-induced pancreatitis or CCK octapeptide-induced pancreatitis in rats^[18,21,22]. We next investigated the effect of PS on HSP60 and HSP72 expressions in CCK octapeptide-induced AP. PS increased pancreatic HSP60 and HSP72 expression in CCK octapeptide-induced AP compared with saline (Figure 3).

Effect of PS on cytokines secretion in CCK octapeptide-induced AP

Various cytokines (TNF- α , IL-1 β and IL-6), released locally and systemically, have been implicated in the inflammatory response associated with pancreatitis. The production of these cytokines has been shown to increase in serum during CCK octapeptide-induced AP^[23-25]. In this study, we found that PS pretreatment significantly decreased the levels of TNF- α , IL-1 β and IL-6 production during CCK octapeptide-induced AP (Figure 4).

DISCUSSION

PS has been used for treatment of various diseases, including edema, appendicitis, endometritis, and inflammation^[16]. On the basis of the idea that the expressions of HSPs, such as HSP 60 and HSP72, in pancreas play a key role in the inflammatory response of pancreatitis, we determined whether PS could increase expression of HSP in CCK octapeptide-induced AP. HSPs play a universal role in the maintenance of cellular homeostasis^[22]. They are expressed constitutively and/or at elevated levels upon the exposure of cells to a variety of stress conditions in every organ, including pancreas^[21,26]. Previous reports of the effects of cerulein-induced AP on pancreatic HSP levels are somewhat conflicting^[27-31]. Ethridge *et al.*^[28] found that the pancreatic heat shock factor 1 (HSP-1) binding activity was increased during cerulein-induced AP, with concomitant increases in HSP70 and HSP27, while the levels of HSP60 and HSP90 were not affected. Similarly, Weber *et al.*^[27] demonstrated that cerulein induced pancreatic HSP70 mRNA and protein expressions. However, secretagogue treatment did not alter the level of ubiquitin mRNA. Using antisense oligonucleotides, Bhagat *et al.*^[29] have shown that cerulein-induced HSP72 expression acts to limit the severity of the disease in rat. Strowski *et al.*^[30] observed that cerulein time- and dose-dependently increased mRNA but paradoxically reduced protein levels of rat pancreatic HSP60 and HSP72. In accordance with their study, we demonstrated that repeated injections of supramaximal doses of CCK octapeptide for 5 d reduced the pancreatic HSP60 and HSP72 levels^[18,32]. However, this decrease was recovered by the administration of the PS. Therefore, PS acted against the effect of CCK octapeptide-induced pancreatitis to decrease the levels of these HSPs, and clearly increased the protection against the disease as discussed below. Since we only examined the quantities of the most widely investigated HSP60 and HSP72, we could not rule out that other HSPs were induced and contributed to the protective effects of PS.

These cytokines (TNF- α , IL-1 β and IL-6) are thought to mediate the systemic effects of pancreatitis, such as fever, hypotension and shock^[8]. Serum levels of TNF- α , IL-1 β and IL-6 are used to evaluate the systemic cytokine response. TNF- α has been shown to be an important initiator of the local and systemic damage occurring in AP^[33]. The overall rise in both tissue and serum TNF- α concentrations correlates directly with the severity of pancreatic damage and inflammation^[34]. Additionally, serum levels of TNF- α have been found to correlate with severity of AP in humans^[24]. IL-1 β is another potent pro-inflammatory cytokine, which, like TNF- α , is derived predominantly from macrophages^[35]. Combined infusions of TNF- α and IL-1 β have synergistic pro-inflammatory effects^[36]. Using knockout (-/-) mice deficient in IL-1 type 1 receptors, TNF type 1 receptors, or both, it has been shown that IL-1 β and TNF- α make an equivalent contribution to the severity of an attack. Preventing the activity of both cytokines concurrently has no additional effect on the degree of pancreatitis^[37].

IL-6, a principal mediator of acute phase response, is primarily released from activated mononuclear phagocytes.

Pooran *et al.*^[38] showed that IL-6 levels in severe pancreatitis were significantly elevated compared with mild pancreatitis^[38,39]. As aforementioned, we demonstrated that PS could reduce TNF- α , IL-1 β and IL-6 production during CCK-induced AP in rats.

Repeated supramaximal doses of CCK octapeptide stimulation for 5 d are known to induce a prolongation of the morphological (increased pw/bw) and biochemical changes (increased serum amylase and lipase activity) in CCK octapeptide-induced acute interstitial pancreatitis in rats^[5-8]. We have found that PS pretreatment reduced typical laboratory parameters (increased pw/bw) and biochemical changes (increased serum amylase and lipase activity) in the CCK octapeptide-induced AP. From these results, we suggest that these cytoprotective effects maybe due to the induction of HSPs. Absolutely decisive proof of the protective effects of HSPs in this AP model would require the blockade of expression or function of these proteins. Unfortunately, there are as yet no specific inhibitors of these.

In conclusion, our study has shown that PS pretreatment ameliorates the severity of CCK octapeptide-induced pancreatitis in rats. In accordance with others, we have demonstrated that PS increases synthesis of HSP60 and HSP72 and reduces the production of TNF- α , IL-1 β and IL-6 in CCK octapeptide-induced AP in rats. Additionally, PS pretreatment ameliorates typical laboratory parameters (increased pw/bw) and biochemical parameters of the disease. Thus, our study suggests that PS protects against CCK octapeptide-induced AP in rats.

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Major complications following exenteration in cases of pelvic malignancy: A 10-year experience

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Abstract

AIM: To analyze the major complications after exenteration of gynecological and rectal malignancies.

METHODS: Twenty-two patients with gynecological malignancy and 6 with rectal malignancy underwent pelvic exenteration (PE) between 1996 and 2005. PE was performed for primary malignancy in 71.4% of cases (vulvar cancer in 13, cancer rectal in 5, cervical cancer in 1 and Bartholin's gland cancer in 1 cases respectively and recurrent malignancy in 28.6% of cases (cervical cancer in 5, ovarian cancer in 1, uterine sarcoma in 1 and rectal cancer in 1 cases respectively). Posterior PE, total PE and anterior PE were most often performed.

RESULTS: Major complications in the operative field involving the urinary tract infection or the wound dehiscence occurred in 12 patients (42.9%). Early complications included massive bleeding from the sacral plexus, adult respiratory distress syndrome (ARDS), thrombophlebitis, acute renal failure, urinary bladder dysfunction, ureter damage, re-operation and pulmonary embolus. Urinary incontinence was observed in 2 women as a late complication. In 1 patient a nephrostomy was performed in 1 patient due to extensive hydronephrosis and 1 patient had complications connected with the gastrointestinal tract. The mortality rate was 7%, of which inter-operative mortality accounted for 3.5%. Major complications often occurred in advanced primary vulvar cancer affecting those with recurrent malignancies.

CONCLUSION: PE is more beneficial to patients with primary vulvar and rectal cancer than to those with recurrent cancer. Knowledge of the inherent complications and morbidity of PE is essential.

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INTRODUCTION

Total pelvic exenteration (TPE) has been used as a salvage therapy. Candidates are those who have failed radiation therapy or primary surgical or combined treatment of the recurrence in the central pelvis^[1-3]. Pelvic exenteration (PE) is also a method of treatment in cases of locally advanced primary pelvic tumors.

PE is carried out at the site of extensive pelvic tumor, and cervical, vulvar, vaginal, ovary, rectal cancer or bladder cancer which can not be removed by standard radical pelvic surgical techniques^[2, 4]. PE may result significant complications, its major complications can affect 62% of patients^[5-9]. The distribution of complications has changed over the years due to the advances in antibiotic therapy and improved supportive care including hyper-alimentation. At present the most threatening complications are those involving the gastrointestinal and urinary systems. Urinary fistulae and obstruction following PE are the frequent and life threatening complications, which increase the mortality and morbidity rates after PE of gynecological cancers^[10].

The present study was to review the literature and report our experience with the complications arising from PE as a radical surgery in the treatment of advanced pelvic malignancies.

MATERIALS AND METHODS

Twenty-two patients with gynecological malignancy and 6 with rectal cancer underwent PE in 1996 - 2005 at the Department of Gynecology, Medical University of Gdansk, Poland. Exenteration was performed because of vulvar cancer in 13 cases, rectal cancer in 6 cases, cervical cancer in 6 cases, ovarian cancer in 1 case, uterine sarcoma in 1 case and Bartholin's gland cancer in 1 case.

The clinical and pathological records were reviewed to determine the primary disease, previous treatment, type

of PE, postoperative morbidity and mortality as well as complications. The primary malignancies (71.4%) included advanced carcinoma of the vulva in 13 cases, rectal cancer in 5 cases, cervical cancer in 1 case, Bartholin's gland cancer in 1 case. The recurrent tumors (28.6%) included carcinoma of cervical cancer in 5 cases, ovarian cancer in 1 case, uterine sarcoma in 1 case, rectal cancer in 1 case.

Posterior pelvic exenteration (PPE), total pelvic exenteration (TPE) and anterior pelvic exenteration (APE) were most often performed.

All the vulvar and cervical cancers were squamous cell carcinoma (SCC). Surgery was performed by a team of gynecological surgeons or together with a team of urologists in cases requiring urinary diversion. All patients were operated under mixed anesthesia consisting of conduction anesthesia (spinal or epidural) and general anesthesia. Closure of the empty pelvic cavity was achieved by mobilization of omentum from the left side of the sigmoid colon or by reperitonization using the mobilized caecum. Re-operation was needed for early and late complications. Early complications were those arising within 30 days after surgery and late complications were those over 30 days after surgery.

RESULTS

The clinical characteristics of 28 patients who underwent pelvic exenteration are summarized in Table 1.

The mean age of the 28 patients at diagnosis was 53.3 years (range, 34-82 years).

The estimated blood transfusion during operation ranged from 240 mL to 3580 mL with a mean of 1100 mL. The operating time ranged from 4 h to 11 h and 45 min with a mean of 6 h and 36 min. Patients stayed 8 - 66 d in hospital after operation with a mean of 27 d.

The overall complication rate after PE was 53.6% (15 of the 28 patients). Wound dehiscence (wd) occurred in 9 cases (32.1%), urinary infection in 3 cases (10.7%), urinary incontinence in 2 cases (7.1%), massive bleeding in 2 cases (7.1%). ARDS, thrombophlebitis, ureter damage, acute renal failure, pulmonary embolism, resuture, and urinary bladder dysfunction occurred in 1 case (3.6%).

The mortality rate was 7%, of which 3.5% was interoperative.

In our study, major complications often occurred in advanced primary vulvar cancer (mainly wound dehiscence), affecting those with recurrent malignancies.

Early complications

Massive bleeding occurred in 2 patients during operation. One patient with uterine sarcoma died of massive bleeding from sacral vessels during PPE although he received 1800 mL blood. The other patient had massive bleeding from the sacral plexus during PPE for vulvar cancer. An attempt was made for haemostasis. Disseminated intravascular clotting occurred and haemorrhage was massive and lasted for a long time. Because of the continuing bleeding and the poor general state of the patient, five large laparotomy sponges were left in the pelvis to cover the pelvic, iliac and sacral vessels under pressure. The emergency pelvic packing was successful and

the sponges were removed after 24 h. Twenty-eight days after the operation, the patient was transferred to the Department of Radiotherapy for supplementary treatment.

Gastrointestinal complications occurred after APE in 1 patient with cervical cancer. During the operation it was impossible to implant the ureter into the ileum by the Bricker method because of a lack of blood supply in the isolated intestinal loop, and uretero-cutaneostomy was performed.

The patient also suffered from acute renal failure as an early complication. Two days after APE the parameters of renal failure decreased gradually and a considerable worsening was found on day 8. Acute renal failure was confirmed. One patient with recurrent cervical cancer underwent APE and died of pulmonary embolism 2 wk after discharge from hospital. Another patient underwent TPE for recurrent cervical cancer and he received re-operation because of bleeding 2 h later. Haemorrhage was identified in the venus plexus surrounding the urethra but not fully achieved and one laparotomy sponge was applied under pressure for 48 h. After removal of the sponge no further bleeding was observed.

Late complications

Late complications occurred as urinary incontinence in 2 patients (7.1%) with vulvar cancer after PPE. A unilateral nephrostomy was performed in 1 patient with vulvar cancer due to extensive hydronephrosis and chronic renal failure one year after PPE.

A urinary fistula was diagnosed 6 mo after PPE for recurrent cervical cancer in 1 patient. During surgery to remove the fistula, a progression of disease was diagnosed and a cystectomy was performed to create an ileal conduit.

DISCUSSION

Primary radiation therapy or surgery in combination with radiotherapy has been the standard treatment for years in patients with advanced cancer. Although some changes have taken place in radiation techniques, the cure rate for advanced cancer still remains disappointing. Severe radiation complications may occur in these patients and PE should be performed for salvage therapy in combination with chemotherapy and radiotherapy as the first line treatment^[5].

TPE is often the only hope for women who have failed non-conservative therapy^[11, 12]. PE can provide a good chance of long-term survival in carefully selected patients but the role of palliative exenteration in patients with non-resectable disease is still controversial^[13]. PE is also the treatment of choice for the control of locally advanced recurrent gynecological malignancies unresponsive to therapy^[4].

We have performed PPE mainly for advanced vulvar cancer without the need to make an ileal conduit in patients who had no previous operation, radiotherapy or chemotherapy.

Physiological age and absence of co-morbidity appear to be more important when patients are selected for exenteration than chronological age. Careful pre-operative staging either by computed tomography scan

Table 1 Clinical characteristics of patients

| Patient number | Age (yr) | Year of operation | Days in hospital after surgery | Histology | Method | Blood transfusion (mL) | Operating time (min) | Complications |
|----------------|----------|-------------------|--------------------------------|------------------------|--------|------------------------|----------------------|---|
| 1 | 59 | 1996 | 48 | vulvar - scc | PPE | 240 | 240 | wd |
| 2 | 48 | 1996 | 14 | vulvar - scc | PPE | 800 | 315 | - |
| 3 | 62 | 1998 | 35 | vulvar - scc | PPE | 600 | 330 | urinary infection, wd |
| 4 | 77 | 1998 | 28 | vulvar - scc | PPE | 900 | 360 | - |
| 5 | 37 | 1998 | 15 | rectal-adenoca | PPE | 600 | 255 | - |
| 6 | 50 | 1999 | 23 | ovarian-adenoca | PPE | 1200 | 405 | - |
| 7 | 65 | 2000 | 54 | vulvar - scc | PPE | 1500 | 380 | urinary infection, wd |
| 8 | 34 | 2000 | 49 | vulvar - scc | TPE | 1200 | 330 | ARDS, wd |
| 9 | 52 | 2000 | 16 | vulvar - scc | PPE | 300 | 360 | urinary incontinence |
| 10 | 44 | 2000 | - | uterine sarcoma | PPE | 1800 | 275 | massive bleeding- died during operation |
| 11 | 43 | 2002 | 28 | vulvar - scc | PPE | 1560 | 350 | - |
| 12 | 64 | 2002 | 28 | vulvar - scc | PPE | 900 | 420 | - |
| 13 | 63 | 2002 | 42 | vulvar - scc | TPE | 1420 | 450 | thrombophlebitis, wd |
| 14 | 53 | 2002 | 25 | Bartholin's gland -scc | PPE | 1100 | 360 | urinary infection, wd |
| 15 | 44 | 2002 | 41 | vulvar - scc | PPE | 1500 | 450 | ureter damage, wd |
| 16 | 64 | 2002 | 14 | rectal-adenoca | PPE | 300 | 315 | - |
| 17 | 62 | 2002 | 16 | cervical - scc | PPE | 500 | 360 | - |
| 18 | 57 | 2003 | 28 | vulvar - scc | PPE | 3580 | 420 | massive bleeding |
| 19 | 34 | 2003 | 24 | cervical - scc | APE | 1285 | 480 | acute renal failure |
| 20 | 34 | 2003 | 8 | rectal-adenoca | PPE | 280 | 380 | - |
| 21 | 74 | 2004 | 66 | vulvar - scc | PPE | 500 | 330 | wd, urinary incontinence |
| 22 | 82 | 2004 | 16 | rectal-adenoca | PPE | 950 | 330 | - |
| 23 | 53 | 2004 | 10 | cervical scc | APE | 1200 | 410 | pulmonary embolism-death |
| 24 | 48 | 2005 | 15 | rectal-adenoca | PPE | 900 | 435 | - |
| 25 | 43 | 2005 | 14 | cervical-adenoca | TPE | 1500 | 660 | wd, bleeding-reoperation |
| 26 | 56 | 2005 | 29 | rectal-adenoca | PPE | 1200 | 705 | urinary bladder disfunction |
| 27 | 51 | 2005 | 26 | cervical scc | PPE | 1200 | 560 | urinary fistula |
| 28 | 38 | 2005 | 10 | cervical scc | TPE | 1800 | 430 | - |
| Mean | 53.3 | - | 26.7 | - | - | 1100 | 396 | - |

Wd = wound dehiscence; ARDS = adult respiratory distress syndrome; SCC = squamous cell carcinoma

or by magnetic resonance imaging can usually identify patients with distant metastases, extrapelvic nodal disease, or disease involving the pelvic sidewall (which generally precludes surgery). Recent application of intraoperative radiotherapy or postoperative high-dose brachytherapy in patients with advanced pelvic disease involving pelvic sidewall, may expand the standard indications for exenteration. However, this procedure with or without radiotherapy, should be the resection of all tumors since the site of palliative exenteration is controversial^[14].

A successful operation can free patients from the potential discomfort caused by aggressive tumor. To a certain extent it can also reduce the pain in the pelvic

area^[6, 13].

The most serious and common complications after exenteration are acute enteric complications (which can exceed 20%), enteric obstruction, fistulization, pelvic infection, sepsis, wound infection, pyelonephritis. Acute renal failure is a rare complication after pelvic exenteration^[7-9,15-18].

Inguinal lymphadenectomy combined with PE increases the total incidence of complications in patients with vulvar carcinoma. Necrosis of the skin over inguinal and symphysis pubis areas is the most common complication which is present in 75% of cases^[18].

The long operating time and huge blood loss associ-

ated with exenteration increase the risk of wound infection which may adversely affect anastomosis site healing. Concomitant transfusion requirements and the entry of contaminated viscera- vagina, urethra, rectum are inherent to the operation. Most patients who undergo the procedure have advanced cancer and have received high-dose radiotherapy to the operative field. This compromises healing ability and makes the procedure even more risky^[4].

Experience shows that irradiation produces relative ischemia of the exposed area with diminished cellular vitality, thereby impairing the healing process^[19]. The dose of previous radiation therapy (especially higher than 4000cGy) is the most important risk factor for major surgical complications. The incidence of postoperative urinary or gastrointestinal complications is significantly higher in previously irradiated gynaecological patients^[4,7,10,20]. Averette *et al*^[15] reported an operative mortality of 40% is associated with surgical correction of fistula and 93% of these patients have received previous radiation therapy. The fistulized loop of bowel is attached to the pelvic floor at reoperation. In our study, fistulization after PE was found only in 1 patient with recurrent cervical cancer. This low incidence can be attributed to the majority of patients presenting primary tumors, who did not undergo primary radiation but reperitonisation at closure of the pelvic cavity to prevent small bowel prolapse. Rodriguez-Bias *et al*^[21] showed that 67% of patients who did not receive prior radiation therapy and 26% of patients who did not receive prior irradiation develop postoperative complications. Other authors have implicated prior radiation therapy as a risk factor for increased morbidity after PE^[15,22].

The type of urinary diversion is also significantly related to the development of complications. A modified Indiana pouch and transverse colon for the reservoir are reported to have a lower incidence of complications than the sigmoid colon or Kock pouch^[10,12,23,24]. Compared to cutaneous ureterostomy and ileal conduit, a continent reservoir provides a better quality of life and a low incidence of pyelonephritis and chronic renal failure. However, the early complication of wound infection is higher^[4].

Urinary fistulae and obstruction following pelvic exenteration are the frequent and life threatening complications, which increase the mortality and morbidity rates of large resections performed during PE for gynecological cancers. Major early urinary complications are significantly increased in patients who have received previous pelvic radiotherapy or have had an intestinal conduit for urinary diversion. Late complications are associated with urinary diversion, including stenosis, chronic or recurrent pyelonephritis, prolapsed stoma, incontinent or obstructed reservoir and calculi in the reservoir^[10].

Patients after PE are at high risk of developing cardiac complications, ARDS and pulmonary emboli^[25,26]. Contrary to other authors we have performed PPE mainly in advanced vulvar cancer without the need to make an ileal conduit in patients did not receive radiotherapy or chemotherapy. The majority of these patients had recurrence after prior surgery and radiotherapy. After exenteration, the 5-year survival was 40-60% in patients with gynecological cancer and 25-40% in patients with colorectal cancer^[14]. We present a review of complications and their

percentages as cited in literature^[3,5,7-9,15-18]

Early postoperative complications

Intestinal obstruction rate was 5.3-21.1%, skin flap necrosis (in vulvar cancer) 75%, hemorrhage 1-16.6%, intestinal fistula 5-16.3%, enterocutaneous fistula 4-23.8%, urinary fistula 1-15.7%, pyelonephritis / pyelonephrosis 3.8-21.6%, wound infection 2-14%, peritonitis 4%, pelvic abscess 2.6-17.9%, stoma separation 5.8%, ureteral obstruction or necrosis 1-5%, uremia (without obstruction) 5.7%, stoma stenosis 2%, prolonged ileus 15%, postoperative psychosis/depression 1.9-5%, pelvic cellulitis 7.4%, stomal hernia 10.5%, colostomy necrosis 2.4-5.2%, loop necrosis 3.7%, iliac artery thrombosis 0.4-1.1%, arterial thrombosis 2.4%, hydronephrosis 15.4%, hyperchloremic acidosis 7.4%, thrombophlebitis 2-8.3%, pulmonary embolus 2-4.3%, neurogenic bladder 8.3%, urinary incontinence 5.3%, cerebrovascular incident 2.2%, shock 3.3%, calculi 2%, myocardial infarction 2.2%, heart failure 0.9%, perineal evisceration 2- 6%, metabolic disorders 21%, pneumonia 1%.

Late postoperative complications

Intestinal obstruction rate was 4.4-15.4%, small bowel ileus 10%, hydronephrosis 1.4-21.6%, enteroperineal fistula 1-5%, pyelonephritis 2-10.5%, colostomy necrosis 5%, perineal abscess 1-3.4%, perineal hernia 1.9-3.3%, renal calculus 0.9-6.2%, stomal hernia 3.7-5%, stomal stricture 1-5.5%, uretero-ileal stricture 1-9.2%, recurrent infection 27.2%, small bowel fistula 5.5-8%, wound dehiscence 9.8%, urinary incontinence 8.3%, chronic lymphoedema 16.6%, perineal evisceration 4%, metabolic disorders 8%, urinary fistula 3%.

Robertes *et al*^[9] reported that 29% of patients after PE need re-operation. Re-operation after PE is extremely difficult and often leads to further morbidity if not mortality in such a situation. Re-operation for small bowel fistula and obstruction has 40% and 50% operative mortality, respectively. In our study, only 1 out of 28 patients (3.6%) had indications for re-operation.

PE is a high-morbidity procedure and its major complications correlate with preoperative pelvic radiotherapy and previous pelvic surgery. However sufficient postoperative nutrition (hyper-alimentation), antibiotics and antithrombotic therapy, use of tissue less affected by radiation (such as transverse colon, jejunum) and the creation of a continent reservoir can decrease the incidence of complications and improve the quality of life after this radical procedure^[4].

Although significant advances have been made in radiotherapy and chemotherapy, PE still remains an important part of the armamentarium of pelvic surgery and is the primary and occasionally the only treatment for the control of advanced malignancies.

In conclusion, PE should be considered as the treatment of choice for the control of locally advanced primary and recurrent pelvic malignancies unresponsive to therapy. An understanding of post-exenteration morbidity and complications is necessary. We are continuing to revise and update the procedures to minimize complications and increase survival.

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RAPID COMMUNICATION

ABH and Lewis antigen distributions in blood, saliva and gastric mucosa and *H pylori* infection in gastric ulcer patients

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CONCLUSION: ABH and Lewis blood group antigens are a good indicator for cellular alterations in the gastric epithelium.

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Key words: *Helicobacter pylori*; Gastric ulcer; ABH and Lewis blood groups

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Abstract

AIM: To investigate the ABH and Lewis antigen expression in erythrocytes, saliva and gastric epithelium, as well as the association between *H pylori* and the presence of gastric epithelial lesions.

METHODS: The distribution of ABH and Lewis blood group antigens in erythrocytes, saliva and gastric mucosa of *H pylori*-infected gastric ulcer patients was analyzed. Forty-two patients with gastric ulcer were studied, and fifty healthy individuals were used as control group. The blood group antigens were determined by direct hemagglutination, dot-ELISA and immunohistochemical methods in erythrocytes, saliva and gastric mucosa specimens, respectively. Diagnosis for *H pylori* infection was performed by conventional optical microscopy and ELISA.

RESULTS: A higher seroprevalence of IgG *H pylori* specific antibodies was observed in gastric ulcer patients (90%) compared to the control group (60%). We observed a significant increase of phenotypes O, A₂ and Lewis b in *H pylori*-infected patients. The expression of these antigens had progressive alterations in areas of ulcerous lesions and intestinal metaplasia.

INTRODUCTION

The presence of *H pylori* in gastric mucosa is associated with chronic active gastritis and more severe gastric diseases, including chronic atrophic gastritis, peptic ulcers, stomach cancer, and lymphoma^[1, 2]. However, only a minority of *H pylori*-infected patients develop gastric diseases. In order to explain this fact, the influences of additional factors such as the genetic predisposition of the host and the genotype of *H pylori* strains have been analyzed^[3]. Biochemical studies^[4, 5] have discovered a blood group antigen binding adhesin (BabA), which can mediate bacterial adherence to epithelial cells and seems necessary for *H pylori* pathogenicity by facilitating the subsequent action of the other virulent factors such as VacA and CagA. Borén *et al*^[6, 7] demonstrated that the receptors for *H pylori* on gastric epithelial cells are the H and Le^b antigens of the ABH and Lewis (Le) blood group systems. It has been known for decades that individuals of O blood group phenotype have a higher risk of developing duodenal ulcers^[8, 9] and also a higher incidence of gastric ulcers^[8, 10]. In ulcer disease patients infected with *H pylori* little is known about the presence of ABH and Lewis antigens in erythrocytes, saliva and gastric epithelium. However, alterations in these blood group antigen expressions have been extensively described in stomach cancer and precursor lesions^[11, 12]. This study was to investigate the ABH and Lewis antigen expression in erythrocytes, saliva and gastric epithelium

Table 1 Distribution of ABO and Lewis phenotypes in erythrocytes and saliva of gastric ulcer patients and controls

| Lewis erythrocytes | Saliva | | Secretor | Lewis phenotypes Blood/Saliva | ABO Phenotypes | | | | | | | | | |
|--------------------|-----------------|-----------------|----------|-------------------------------|----------------|----|----------------|----|---|----|----|----|----|----|
| | Le ^a | Le ^b | | | A ₁ | | A ₂ | | B | | AB | | O | |
| | | | | | I | II | I | II | I | II | I | II | I | II |
| Le (a+ b-) | + | + | S | Concordant | 1 | 2 | 1 | - | - | 1 | 1 | - | 2 | - |
| Le (a- b+) | + | + | S | Concordant | 1 | 7 | 2 | 2 | 1 | 7 | - | 1 | 12 | 20 |
| Le (a- b-) | - | - | S | Concordant | - | 2 | - | - | - | - | - | - | 2 | - |
| | + | + | S | Discordant | 3 | - | 1 | 1 | 4 | - | - | - | 7 | 7 |
| | - | + | S | Discordant | 1 | - | - | - | 1 | - | - | - | 2 | - |
| Total | | | | | 6 | 11 | 4 | 3 | 6 | 8 | 1 | 1 | 25 | 27 |

I = gastric ulcer patients, II = control, S = secretor status

in *H pylori*-infected patients as well as the association of *H pylori* status with these blood group phenotypes and the presence of gastric epithelial lesions.

MATERIALS AND METHODS

Patients and control sample

The study included a total of 42 patients with gastric ulcer who were examined by routine upper endoscopy at Ofir Lioila Hospital (Belém, PA, Brazil) between May and December 2000, and comprised 76% males (32/42) and 24% females (10/42). The mean age was 53 years, ranging 28-80 years. Blood and saliva samples and gastric biopsy specimens were collected from each patient. These selected patients did not take non-steroidal anti-inflammatory drugs, H2 receptor antagonists, proton pump inhibitors or antimicrobial drugs for at least 60 d before the samples were obtained. Peripheral blood and saliva samples were collected from 50 patients asymptomatic for gastric diseases. These patients did not receive upper endoscopy. The mean age of these individuals was 49 years, ranging 25 - 80 years. This study was approved by the Ethics Committee at the Tropical Medicine Nucleus of the Pará Federal University and informed consent was obtained from the patients before sample collection.

Histopathological analysis of gastric biopsies

For histological analysis, biopsies from the ulcer lesion border and the adjacent area (perilesion) of each patient were obtained. Paraffin-embedded biopsy specimens were sectioned at 4 µm thickness and stained with haematoxylin - eosin and evaluated using the Sydney classification^[13] with regard to the presence of intestinal metaplasia (IM) and the degree of granulocytic and lymphocytic infiltration (mild, moderate, severe). The density of *H pylori* was determined in the sections using a modified Gram staining and graded into absent, mild, moderate and strong, based on the above classification system^[13].

Serological detection of specific antibodies against *H pylori* and *CagA*

The serum samples were tested for IgG-class antibodies against *H pylori* by an indirect hemagglutination assay and anti-*CagA* with a commercial kit based on recombinant *Helicobacter* antigens p120 EIA. Both tests were performed according to the manufacturer's instructions (Viva

Diagnostika, Hürth, Germany). *H pylori* infection diagnosis of the control group was performed using only serological methods. However in the ulcer disease patients *H pylori* status was determined by serological and conventional optical microscopic methods.

Detection of ABO and Lewis blood group antigens

Blood and saliva samples were collected after the endoscopy. In blood the ABO and Lewis phenotypes were determined with a conventional direct hemagglutination technique. The characterization of ABH and Lewis specificities in saliva was tested using the dot-ELISA technique on nitrocellulose^[14]. Immunohistochemistry for ABO and Lewis blood group antigen expression in gastric biopsies (ulcer lesion border and perilesion) in the foveolar and fund epithelium was performed using an indirect immunoperoxidase technique^[15]. The reaction pattern of these antigens in gastric mucosa was classified as positive (homogeneously with more than 50% of stained cells or heterogeneous with 5-50% of stained cells) and negative (without or lower than 5% of stained cells).

Statistical analysis

Statistical tests using Bioestat 3.0 were performed to verify the significance of the differences observed in our study^[16]. The chisquaretest (χ^2) was used as a global test for any relationship. The Mann-Whitney U test was used to compare unpaired data. Spearman's rank and contingency correlation tests were used to examine the relationship between density of *H pylori*, polymorph nuclear activity and chronic inflammation. $P < 0.05$ was considered statistically significant.

RESULTS

Seroprevalence of *H pylori* infection and *CagA* strains

Endoscopic diagnosis of patients with gastric ulcer indicated 74% (31/42) ulceration in the antral region and 26% (11/42) in the corpus region. The presence of IgG *H pylori* specific antibodies was observed in 90% (38/42) of all patients. Approximately 84% (32/38) of these *H pylori*-infected patients were also *CagA* seropositive. In the control group, seroprevalence of IgG *H pylori* specific antibodies was observed in 60% (30/50) of the individuals and 36% (18/50) were also infected with *CagA* strains. These distributions were significantly lower in *H pylori*-infected patients than in gastric ulcer patients ($P < 0.01$).

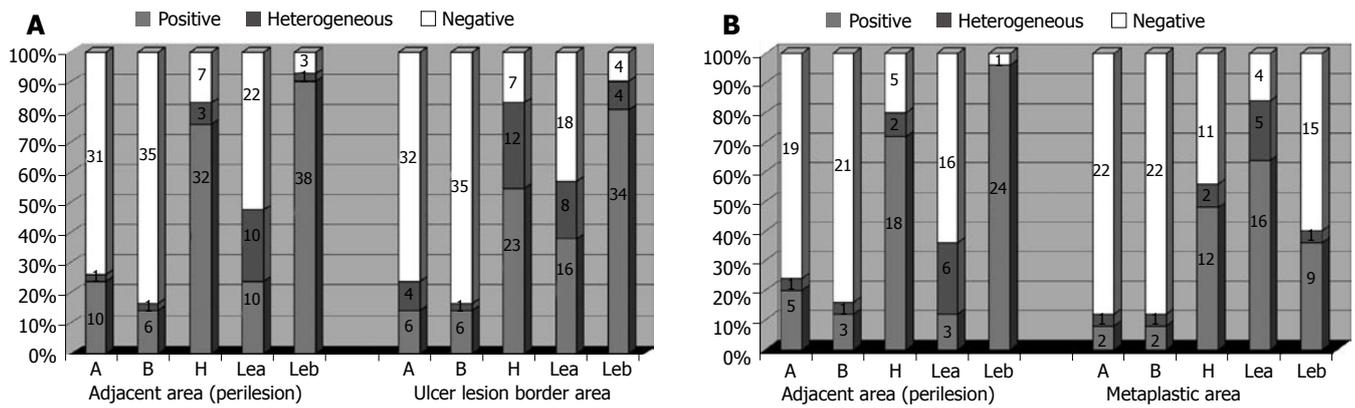


Figure 1 ABO and Lewis antigen distribution pattern in ulcer lesion borders (A) and intestinal metaplastic area (B) of gastric ulcer patients.

Distribution of ABO and Lewis phenotypes in erythrocytes and saliva of patients and control group

A comparison of the ABO blood group phenotype distributions in blood and saliva of gastric ulcer patients and control group is showed in Table 1. Regarding the Lewis blood group system in saliva, the Le^b antigen was detected in approximately 95% of the patients and in the control group. The distribution frequency of ABO ($P>0.05$) and Lewis saliva ($P>0.05$) phenotypes observed in gastric ulcer patients had no difference in relation to the control group. We observed a discrepancy in the expression of Lewis antigens in erythrocytes and saliva in some patients (Table 1), which were divided in two groups: concordant (individuals with similar expression of Lewis antigens in blood and saliva) and discordant (individuals with different expressions of Lewis antigens in blood and saliva). A discordant phenotype was observed in 57% (24/42) of the patients. Of these, 83% (19/24) belonged to the negative erythrocyte Lewis phenotype. A difference in the blood and saliva Lewis antigen expression (Table 1) was observed in only 22% (11/50) of the control group.

Distribution of ABH and Lewis antigen expression in gastric mucosa regions

The pattern of ABH and Lewis antigen distribution in the foveolar epithelium of the perilesion areas compared with ulcer lesion borders showed a loss of A, H and Leb antigen expression, resulting in a decrease of homogeneous expression and an increase in the heterogeneous pattern. At the same time, an increased expression of Le^a antigen was observed in the ulcerous lesion border areas (Figure 1A). The presence of incomplete intestinal metaplasia (IM) was observed in 25 out of 42 ulcer patients. In these patients the same abnormal pattern of ABH and Lewis blood group antigen expression was found to be more intensive, with a significant increase ($P<0.01$) in the expression of Le^a antigen and a decrease in A, H and Le^b antigen expression (Figure 1B).

Correlation of ABH and Lewis blood group antigen expressions in saliva, erythrocytes and foveolar epithelium of gastric mucosa

In the perilesion area of the foveolar gastric epithelium,

Table 2 Distribution of ABO and Lewis blood group phenotypes in relation to serological diagnosis of H pylori infection in ulcer patients and controls

| Blood group phenotypes | <i>H pylori</i> infection | | | | | |
|---|---------------------------|-----------------------|----------|-----------------------|----------|----|
| | N | Patients ^a | | Controls ^b | | N |
| | | Positive | Negative | Positive | Negative | |
| A ₂ Le(a-b+) ou | 27 | 26 | 1 | 30 | 25 | 5 |
| O Le(a-b+) | | | | | | |
| A ₁ /AB/B/Le(a-b+) ou O Le(a-b-) | 15 | 12 | 3 | 20 | 5 | 15 |
| Total | 42 | 38 | 4 | 50 | 30 | 20 |

^a $P>0.05$ vs bacterial density, lymphocytic and granulocytic infiltration

the ABH antigen expression was in agreement with that in erythrocytes and saliva. All the patients belonging to the A blood group (10/42) expressed antigens A and B. Six of them also expressed antigen H. Likewise, patients of the B blood group (a total of six) expressed antigen B, antigen H was expressed in four of them. Patients of AB blood group expressed antigens A and B. Antigen H was expressed in all O blood group patients (a total of 25 individuals). Analysis of the Lewis phenotype in the perilesion region showed a similar antigen expression pattern to that in saliva of all patients, including those with discordant Lewis phenotypes in saliva and blood. Only one patient had discordant and concordant expression with the erythrocyte phenotype.

Association of ABH and Lewis blood group antigen distribution with H pylori infection and histopathological findings

The Lewis saliva phenotype was used to associate *H pylori* status and the development of gastric ulcers, because this expression was similar to that in the foveolar epithelium in gastric biopsies. Furthermore, the presence of ABO and Lewis antigens in the control group was determined only in saliva and erythrocytes but not in gastric mucosa. In relation to the seroprevalence of *H pylori* infection, a significantly higher level was observed in a defined combination of O-/A₂ Le (a-b+) blood groups than in the set of other blood group phenotypes of the control group (Table 2). However, no significantly different proportions were found in this subdivision, a finding that

might be explained by the high prevalence of the infection. A significant positive correlation was found between bacterial density and degree of chronic inflammation ($P < 0.05$) as well as the polymorph nuclear activity ($P < 0.05$). The degree of chronic inflammation was found to be positively correlated with polymorph nuclear activity ($P < 0.01$). Subsequently, no significant correlation was found by contingency analysis for the variability of histological scores in regard to bacterial density between O-/A₂ Le (a-b+) individuals and the set of other blood group phenotypes ($P > 0.05$), lymphocytic ($P > 0.05$) and granulocytic infiltration degrees ($P > 0.05$) in biopsies from ulcer patients.

DISCUSSION

The sero-prevalence of IgG *H pylori* specific antibodies is 90% in patients with gastric ulcer, much higher than in asymptomatic individuals (60%). Additionally, the *cagA* seropositive phenotype shows a significant association with gastric ulcer patients. The frequencies obtained in patients agree with the reported results in previous studies^[17], in which a high rate of infection was found in symptomatic adults. The same occurs with the rate obtained in asymptomatic adults. This fact also corroborates the studies of seroprevalence in developing countries, which describe a 60% infection rate in the adult populations^[18, 19]. The Lewis blood group antigens in saliva and blood of patients and the control group demonstrated a high frequency of discordant Lewis phenotype. Among these individuals, most of them were grouped in the non-genuine negative Lewis phenotype according to the Ørntoft classification^[20], where Lewis antigens are present in saliva but not in the erythrocytes, which is different from the genuine Lewis phenotype, in which the Lewis antigens are present neither in saliva nor in erythrocytes. The expression of ABH and Lewis blood group antigens in the foveolar epithelium in perilesion areas was similar to that observed in saliva, with no differences in relation to the normal synthesis of these antigens. This can be explained by the fact that circulating Lewis antigens in the serum are only acquired by red cell membranes^[21]. In some physiological conditions and diseases, such as neoplasia, a reduction in the synthesis of these antigens can occur, so that the quantities of these antigens in the blood are not sufficient to be detectable by serological methods, however the salivary phenotypes do not alter^[21]. Probably, the increased degree of inflammation in the gastric mucosa due to infection with *H pylori*, affects the metabolism of glycoconjugates, leading to a decrease in the quantity of Lewis antigens circulating in the plasma. The ABO blood group phenotype frequencies in ulcer patients and the control group were not different. This finding is in contradiction to many studies, which have described the increased prevalence of ulcers among O group individuals^[9, 10].

An interesting finding of our study is the observation that all A₂ subgroup individuals were *H pylori* positive, which has been described in another study regarding gastritis patients^[18]. The reason for this finding is not clear. Theoretically, the qualitative structural difference between A₁ and A₂ subgroups is based on glycosphingolipids ex-

pressed in erythrocytes, so that A₁ transferase is more efficient for converting specific carrier types (H type 3 chains or H type 4 chains) into a determinant than A₂ transferase, resulting in a higher level of H epitope in A₂ subgroup, almost like that in O blood group^[20]. Therefore, we believe that analogue fucosylated structures might exist as a mucosal gastric component relating to the different A subgroups, since glycosphingolipids present in gastric mucosa can function as receptors for the bacterium^[22]. Besides, complex gangliosides (i.e. repetitive N-acetylglucosamine units, fucose branches and di- or multivalency) have been demonstrated in the binding assays as preferred structural requirements for mediating *H pylori* adherence to gastrointestinal epithelial cells^[23].

Furthermore, our study regarding ABO and Lewis phenotype and *H pylori* infection indicated that individuals with O Le (a-b+) and A₂ Le (a-b+) phenotypes had a tendency towards a higher rate of *H pylori* infection. Studies performed by Bóren *et al.*^[6, 7] in 1993 and 1994 have suggested that *H pylori* uses carbohydrate structures with terminal fucose as receptors in the gastric mucosa containing Le^b and H blood group specificities. Later on, Ilver *et al.*^[5] in 1998 and Gerhard *et al.*^[24] in 1999 showed that there is a higher susceptibility to *H pylori* infection in individuals of O and Le^b blood groups, because they have a higher quantity of fucosylated antigens.

In the current study, the association of ABH and Lewis blood group phenotype distributions with the histological scores was not significantly different. But if one considers that Le^b is absent only in 2 out of 42 patients, this result is inappropriate for comparison with other histopathological studies^[25-27]. By comparing the ABH and Lewis antigen expression between the ulcerous lesion border and the adjacent areas (perilesion), we observed an increase in the heterogeneous expression pattern, demonstrating the loss of A, H and Le^b antigen expression and appearance of Le^a reactivity in inflammation regions. Colonization of the gastric mucosa by *H pylori* is a relevant factor that can alter the normal pattern of homogeneous expression.

Some studies have demonstrated that the expression of these blood group antigens is altered in metaplastic areas^[17, 28]. The main alterations described are the increase of Le^a and the decrease of H and Le^b antigens, as was also found in this study. One explanation for this observation is the repression of the secretor enzyme activity, leaving more type I precursor chains available to be transformed into Le^a by the Lewis enzyme, consequently reducing the expression of the H antigen and its transformation into A and/or B and Le^b antigens in these tissues^[29].

In conclusion, the rate of *H pylori* infection seems to be higher among O, A₂ and Le(a-b+) phenotype individuals. The pattern of Lewis expression changes in ulcer disease patients with *H pylori* presence, mainly in intense inflammation and/or intestinal metaplastic areas, showing the increase of Le^a and loss of H and Le^b antigens in the gastric mucosa. Therefore, it is important to understand how ABH and Lewis antigens are regulated in gastric cancer, as well as the interaction of these histo-blood group antigens with *H pylori* adhesion, which needs to be further investigated.

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Effect of oral naltrexone on pruritus in cholestatic patients

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CONCLUSION: Naltrexone can be used in the treatment of pruritus in cholestatic patients and is a safe drug showing few, mild and self-limited complications.

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Key words: Cholestasis; Pruritus; Naltrexone

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Abstract

AIM: To determine the efficacy and potential complications of oral naltrexone used in the treatment of pruritus in cholestatic patients and to compare them with other studies.

METHODS: Thirty-four enrolled cholestatic patients complaining of pruritus were studied. In the initial phase, pruritus scores during day and night were evaluated. Subsequently, patients were given a placebo for one week followed by naltrexone for one week. In each therapeutic course (placebo or naltrexone) day and night pruritus scores were distinguished by a visual analogue scale (VAS) system and recorded in patients' questionnaires.

RESULTS: Both naltrexone and placebo decreased VAS scores significantly. Naltrexone was more effective than placebo in decreasing VAS scores. Both day and night scores of pruritus decreased by half of the value prior to therapy in thirteen patients (38%). Daytime pruritus improved completely in two patients (5.9%), but no improvement in the nighttime values was observed in any patient.

Sixteen patients (47%) suffered from naltrexone complications, eleven (32%) of them were related to its withdrawal. Complications were often mild. In the case of withdrawal, the complication was transient (within the first 24-28 h of therapy) and self-limited. We had to cease the drug in two cases (5.9%) because of severe withdrawal symptoms.

INTRODUCTION

Pruritus is one of the most annoying symptoms in cholestatic hepatic diseases^[1-3]. Several therapeutic methods with varying degrees of success have been used in its treatment. However, although we can not ignore the positive effects of cholestyramine^[4], urodeoxy colic acid^[5-7], rifampin^[8-12] and antihistaminic agents on pruritus, many patients do not show unequivocal response to any of these therapeutic options. Even liver transplantation has been indicated in patients with refractory pruritus^[13-15]. The classic explanation for pruritus during cholestasis is the accumulation of bile acids^[16, 17]. However, recent studies show that endogenous opioids in the central nervous system have a role in creating the feeling of pruritus in these patients^[18-20]. Plasma levels of endogenous opioids including enkephalin increase in patients with chronic cholestasis^[21]. Pruritus can be controlled by opioid antagonists such as naloxone^[22, 23]. Injection of cholestatic patient's serum to monkey's medulla can cause pruritus that is controlled by naloxone^[24]. Several recent studies indicate that opioid antagonists such as naloxone and nalmefene are effective in reducing pruritus in patients with primary biliary cirrhosis^[18, 25-27]. Unfortunately, these drugs have some limitations in use. Naloxone has a short half-life and low bioavailability. Therefore, the only way to use it is by injection^[27, 28]. Furthermore, these opioid antagonists frequently cause severe withdrawal reactions in patients^[29-31].

Naltrexone is an oral opiate antagonist that is commonly used in reducing alcohol dependence and opioid addiction^[28, 32, 33]. It has been used more recently for rapid opiate detoxification^[34]. Acute naltrexone withdrawal reactions have also been reported^[35]. Its half-life and bioavailability are between naloxone and nalmefene and it has a considerable first pass effect (95%). Naltrexone clearance from serum is mostly in kidneys^[28, 36, 37]. We performed this study to evaluate the effects of naltrexone on cholestatic pruritus and its complications.

MATERIALS AND METHODS

Thirty-four patients (age range: 32-72 years; average age: 54 ± 11.34 years) with cholestatic pruritus were selected for study. These patients had different types of cholestatic diseases including primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC), cirrhosis (in compensated stage), overlap syndrome, choledocholithiasis, cholangiocarcinoma, periampullary tumors and pancreatic head carcinoma. The duration of pruritus ranged from eight days to thirteen months. Criteria for exclusion of patients from the study included age less than 15 years, serum creatinine >1.5 mg/dL, pregnancy, use of opioids, and cirrhosis with B or C Child Paugh score. The study was performed between April 2003 and June 2004 in the Imam Hospital in Tehran, Iran. We conducted the study on admitted patients with the approval of the Ethics Committee of Tehran University of Medical Sciences. All enrolled patients gave their informed consent.

The study was carried out using a single blind, self-controlled trial method. After the day and night pruritus scores were obtained, patients were given a placebo for one week and then the scores were recorded. Subsequently, a naltrexone therapy (50 mg daily) for one week, was begun. Pruritus scores were characterized by a VAS system between 0 and 10. Daytime pruritus was considered as pruritus occurring before sleep and nighttime implied pruritus after waking from nocturnal sleep. A score of zero signified no pruritus while 10 meant sleep or work-disturbing pruritus or of such severity that skin damage occurred.

Scoring was performed in the middle and at the end of the therapeutic course of treatment (placebo or naltrexone) and the mean of the scores was used. Laboratory tests including total and direct bilirubin, alkaline phosphatase (ALP), aminotransferases (AST, ALT) and creatinine were performed for all patients before therapy and recorded in the questionnaires. The mean scores of day and night pruritus were compared before therapy and after placebo and naltrexone courses by Wilcoxon rank test with SPSS10.0.5 software. $P < 0.05$ was considered statistically significant.

RESULTS

The numbers of patients with PBC, PSC, cirrhosis, overlap syndrome, choledocholithiasis, cholangiocarcinoma, periampullary tumors and pancreatic head carcinoma were 4, 6, 11, 7, 1, 2, 2, and 1, respectively.

Naltrexone effect on pruritus

Table 1 Characteristics of patients before therapy ($n = 34$)

| Characteristics | Range | Mean \pm SD |
|----------------------------------|-----------|---------------------|
| Age (yr) | 32-72 | 53.97 \pm 11.93 |
| ALP | 190-1150 | 657.76 \pm 266.36 |
| Total bilirubin | 5.8-37.00 | 16.60 \pm 9.03 |
| Direct bilirubin | 4-32.5 | 13.14 \pm 8.05 |
| ALT | 32-145 | 66.03 \pm 27.13 |
| AST | 35-110 | 60.47 \pm 19.73 |
| Creat. | 0.7-1.3 | 1.01 \pm 0.14 |
| Pruritus in day before therapy | 6-10 | 8.30 \pm 1.07 |
| Pruritus in night before therapy | 8-10 | 9.13 \pm 0.78 |

The characteristics of patients before therapy are summarized in Table 1. In both cases of therapy with placebo or naltrexone, the mean scores of daytime and nighttime pruritus decreased significantly (Table 2). Naltrexone significantly decreased the pruritus score compared to the placebo (Table 2).

In this investigation, two of the thirty-four patients were forced to withdraw from the study because of drug complications. Thirteen patients (38%) showed at least 50% decrease in their pruritus scores and two (5.9%) became completely free of pruritus. Three (8.8%) showed no change in their scores during the therapy and the score even increased in one case.

The onset of naltrexone therapeutic effects was within the first 48 h of therapy. The decrease in pruritus gradually slowed down and returned to some extent in three patients. There was no obvious change in biochemical parameters after therapy. The bilirubin and ALP levels did not significantly differ before or after therapy (without considering the result of therapy).

Complications

Sixteen patients (47%) suffered from naltrexone complications which were generally mild and improved without additional treatment in the first 2-3 d of therapy. The most common complication was a withdrawal reaction in eleven patients (32.4%). The general and gastrointestinal complications (including dizziness, nausea, vomiting, headache, abdominal cramps, lethargy, weakness, irritability, dry oral mucous membrane and insomnia) were seen in other patients and one patient had also dermatologic complications. Two patients did not finish their therapeutic course because of severe withdrawal reactions. However, pruritus decreased relatively to its initial condition in one of the two patients. Except for the withdrawal reactions, other complications were not sufficiently severe to cause drug cessation.

DISCUSSION

The results of this study indicate that oral naltrexone, an opioid antagonist, can reduce or improve cholestatic pruritus. The results agree with the other reports^[38-41]. Some researchers believe that the VAS system for evaluating pruritus severity is not reliable and prefer to use a mechanical instrument attached to fingers to show and record patients' pruritus^[25]. Although such instruments may be helpful

Table 2 Comparison of pruritus scores in patients before and after placebo and naltrexone therapy (mean \pm SD)

| Patients (n = 34) | 1 Before therapy | 2 After placebo | 3 After therapy | 1 and 2 Significance | 1 and 3 Significance | 2 and 3 Significance |
|----------------------|---------------------|--------------------|--------------------|-------------------------|-------------------------|-------------------------|
| Pruritus in day | 8.30 \pm 1.07 | 7.54 \pm 1.38 | 4.91 \pm 2.56 | P < 0.001 | P < 0.001 | P < 0.001 |
| Pruritus in night | 9.13 \pm 0.78 | 8.29 \pm 1.02 | 5.54 \pm 2.51 | P < 0.001 | P < 0.001 | P < 0.001 |

in evaluating pruritus objectively, their use has some difficulties. On the other hand, the VAS system can control pruritus quite well^[4, 8-12, 42]. It was reported that the pruritus index is significantly correlated with the pruritus score obtained by the VAS system^[25, 26]. It has been shown that decreased pruritus index and its perception are similar^[43].

In three patients of the present study, the rate of pruritus score decrease was lower after a few days of therapy. The reason is unclear though it may be due to secondary adaptation to opioid or drug resistance (tachyphylaxis). This effect has already been reported in earlier studies on nalmefene^[27, 38, 39, 44].

Some researchers believe that cessation of drug therapy for two days during a week ("drug holidays") can reduce drug adaptation effects in such patients^[38]. However, others consider that this method is ineffective^[40]. Increasing the naltrexone dose to 100 mg/day may be effective in such circumstances. Although side effects were relatively common (47%), most of them were mild, self-limited and transient, requiring no additional therapy in our study. On the other hand, in the placebo group 26% of patients had drug complications. Two patients had to stop the therapy because of severe opioid withdrawal effects. Both of them had a positive opioid addiction history but they were not addicted to it at the time of the study. In comparison with nalmefene, naltrexone leads to fewer and milder complications^[18-27].

To decrease naltrexone complications, synchronous prescription of clonidine^[18] or naltrexone at a low dose, at least for the first few days of therapy^[27], is recommended. Also we can divide the total dose into 25 mg BD instead of decreasing it during the first few days of therapy^[38, 39]. Examples of naltrexone hepatotoxicity have been reported^[45], but there is no report on the hepatotoxicity at the low dose of naltrexone in normal people or patients with hepatic diseases^[38, 39, 46].

Endogenous opioids play a role in producing cholestatic pruritus, but opioid antagonists cannot improve pruritus completely^[27, 38, 39, 47, 48]. Studies indicate that naltrexone is a drug that can be well tolerated by patients and its complications are often mild and transient not requiring additional therapy^[41, 49, 50]. Naltrexone can also be used in treatment of severe and intractable pruritus of varying origins^[41].

In conclusion, naltrexone can be used in the treatment of pruritus in cholestatic patients.

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Effect of special Hungarian probiotic kefir on faecal microflora

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Abstract

AIM: To investigate the effect of a four-week consumption of a special Hungarian probiotic agent (Biofir®) on the faecal microflora in human healthy subjects.

METHODS: The effect of Biofir® with $10^6/\text{cm}^3$ initial germs on the faecal microflora was studied in 120 healthy volunteers (71 females, 49 males). The traditional Russian type kefir was used as control. The various germ groups and pH values were determined in wk 2, 4 and 6.

RESULTS: The number of all microbes increased during the 4-week probiotic treatment. The number of microbes increased 4.3-fold in the control group and 6.8-fold in Biofir-treated group. The probiotic kefir caused multiplication of the probiotic flora, meanwhile the undesired bacteria multiplied in the control group. No significant change of pH values of the faeces was found in both groups.

CONCLUSION: The Hungarian probiotic kefir (Biofir®) is capable of promoting multiplication of probiotic bacterial flora in the large bowel.

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Key words: Hungarian probiotic agent (Biofir); Faecal microflora; Large bowel

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INTRODUCTION

Human intestinal flora contains as many as 10^{14} bacteria classified into 400-500 species, which are ten times higher than all the cells in the human body. Some bacteria of the intestinal flora such as Clostridia, Proteus and Pseudomonas aeruginosa can be harmful, while others like bifidobacteria and Lactobacilli belonging to the so-called probiotic strains are favorable for the organism. The microflora in the large intestine plays an important part in the life of the host organism^[1, 2]. Its composition may change several times during our life. However it can still be regarded as nearly constant. Non-pathogenic, pathogenic and potentially pathogenic microorganisms living in a state of equilibrium determined by their own ecosystem within the large intestine take part in the local immunological and metabolic processes as well as in those affecting the organism as a whole^[3, 4].

Some strains produce metabolites, such as short chain fatty acids and bactericins, which are of antibacterial effects. As a consequence of the relationship between cells of the mucous membrane and the microflora, the expression of certain mucosal genes may change, the cytokin release may increase, the proliferation of mucous membrane may change and produce a significant effect on the intestine-associated lymphoid tissue which is the largest immune organ of the organism containing 80% of cells producing antibodies^[5-9]. Certain strains of bacteria are capable of improving the barrier function of the mucous membrane and increase the differentiation of B cells as well as IgA secretion^[4, 6].

The increasing data raise the idea of enriching foods with probiotics, prebiotics and symbiotic, a mixture of these two. Probiotics which are beneficial non-pathogenic bacteria live in the intestinal canal and play a role in the preservation of health^[10, 11]. The majority of prebiotics are oligosaccharides, i.e. the indigestible constituents of our plant food, which promote the multiplication, growth and efficacy of the strains of probiotic bacteria in the large intestine. Nowadays several strains of probiotic bacteria are known, but their utilization is restricted by the fact that an effective probiotic is supposed to proceed along the acidic pH of the stomach and is able to resist the digestion of bile and pancreatic juice and finally sticks to the surface of some cells in the intestinal wall. The lifespan of the stuck probiotic is short, ranging from a few days to a few weeks and it usually lasts for a short period of time following its regular intake. Colonizing probiotics compete with other microorganisms for nutrients and appropriate binding sites. Only probiotics capable of colonizing even if only temporarily, can exert the required immunological effect^[1, 7, 8].

Table 1 Composition of kefir

| Components | Kefir: control product | Biofir |
|--|------------------------|--------|
| Fat content | 3.5% | 3.5% |
| Prebiotic content | - | 0.4% |
| Number of microbes x 10 ⁶ Cfu | | |
| Lactobacilli | 0.15 | 1.48 |
| Streptococci | 165.00 | 227.50 |
| Yeasts | 0.40 | 0.18 |

The *in vitro* efficacy of the well-known probiotic strains has already been proved in clinical practice^[12]. The most widely used strains are those taken from sour dairy products and the intestinal system. The most frequently studied species include various species of Lactobacilli, Streptococcus, thermophilus, bifidobacteria, Saccharomyces boulardii, but under certain conditions other strains in the intestinal microflora, such as *Escherichia coli* (*E. coli*) can also be used as probiotics^[13,14]. In order to become suitable for producing health improving foods, namely functional foods, individual probiotic strains need to meet certain requirements^[15,16]. The following criteria are listed for effective probiotic bacteria^[17,18]: maintenance of the biotic potential, good taste and flavor following fermentation, mild acidity in the course of storage, preservation of the capability of colonizing in the course of food technology and storage, high degree of stability during storage, stability in the course of freeze-drying or other drying procedures, accurate and reliable determination of the strain, and the dose-dependent effects

Biofir[®] is a traditional, so-called Russian type of kefir based on probiotic lactic acid bacteria culture composed of termophilic strains (producing exopolysaccharides) developed by the Hungarian Dairy Research Institute. The present study was undertaken to investigate the effect of a four-week-long consumption of Biofir[®] on the faecal microflora.

MATERIALS AND METHODS

Patients

One hundred and twenty healthy volunteers (71 females and 49 males) were included in the study following the permission of the Ethical Committee. Their age ranged from 18 to 59 years. The basic selection criterion was that no antibiotics were taken by the subjects 2 mo prior to the investigation.

Composition of kefir (Table 1)

Kefir containing 3.5% of fat was made by stirring the frozen kefir culture. The initial number of cells was 10⁶/cm³. Biofir[®] containing 3.5% of fat was a mixture of curd kefir made by stirring the symbiolact-1 culture. The ratio of ingredients was 1:1. The initial number of cells was 10⁶/cm³.

Prior to the investigation, blood, urine and faeces samples were taken from the subjects for routine laboratory analysis and faecal microflora examination. The investigations lasted for 6 wk, while the subjects followed standard diet which was free of sour dairy products and other probiotic foods. The diet was controlled regularly by the

Table 2 Germ groups and cultures used in study

| Germ groups | Cultures |
|-------------------|--|
| All germs | Plate count skim milk agar |
| Streptococci | M-17-agar according to Terzaghi |
| Lactobacilli | MRS-agar De Man, according to Rogosa and Sharpe |
| Bifidobacteria | MGLP modified Garcke agar |
| Bacteroids | Anaerob blood agar base according to CDC |
| Coliforms | Violet red bile (VRB) agar |
| Escherichia coli | Lauryl sulfate broth |
| | Eosin methylene-blue lactose sucrose (EMB) agar |
| Enterobacteria | Violet red bile dextrose (VRBD) agar according to Mossel |
| -Lactose-positive | Hektoen enteric agar |
| Lactose-negative | |
| Enterococci | Citrate azide tween carbonate (CATC) agar base |
| Anaerob spora | Reinforced clostridial agar |
| | Anaerobic agar according to Brewer |
| Yeasts and moulds | Yeast extract glucose chloramphenicol (YGC) agar |

dietitian. Then the control group (60 persons) consumed 0.5 L of Russian type kefir daily for four weeks, while those included in the study group (60 persons) consumed a daily amount of 0.5 L of probiotic kefir.

Faeces samples were taken in the 2nd, 4th and 6th weeks. The screening type of blood sample analysis was repeated in the 4th wk. Faeces samples were stored in sterile containers at 4 °C and sent to the Hungarian Dairy Research Institute in 4-6 h, where determination of the various germ groups was carried out in internationally accepted cultures. In addition to the analysis of the microflora, the pH value of the faeces was also determined (Table 2).

Statistical analysis

Evaluation of the data included calculation of the changes in percentage. Determination of the differences with regard to the self-control was carried out using one sample T probe, while two-sample T probes were used for the comparison of the two groups.

RESULTS

The essential question in the course of analyzing the faecal microflora was how the consumption of each type of kefir influenced the number of primary probiotic Streptococci, Lactobacilli and bifidobacteria within the total number of germs.

The effect of the tested dairy products on the essential faecal microflora during the 4-wk clinical investigation is listed in Table 3.

The results indicated that the number of all microbes increased 4.3-fold in the control group consuming the traditional Russian type of kefir and 6.8-fold in the group consuming the probiotic Biofir[®], respectively after 4 wk. A very important difference was found between the two groups. The rate of probiotic microbes decreased from 8.9% to 2.7% in the control group by the end of the 4th wk and increased from 12.7% to 72% in the target group. Within the total number of the probiotic germs, the greatest increase (59.7-fold) resulting from the consumption of Biofir[®] could be observed in bifidobacteria, a lower increase (6.8 fold) was found in Streptococci, while the index

Table 3 Effect of consumption of tested dairy products on the essential microflora in 4-wk clinical investigation

| Dairy product consumed | Number of subjects | Germ group | Unit | Germ values of faeces samples | | |
|-----------------------------------|--------------------|---------------------------------------|--------------------|-------------------------------|--------------------|----------------|
| | | | | wk 0 | wk 2 | wk 4 |
| Traditional Russian type of kefir | 15 | Total germ number (Aerobe + anaerobe) | 10 ⁶ /g | 10 585 ± 8350 | 7876 ± 4272 | 45 125 ± 19463 |
| | | | Index | 1.0 | 0.7 | 4.3 |
| | | -Streptococcus | 10 ⁶ /g | 220 ± 105 | 374 ± 179 | 285 ± 123 |
| | | | Index | 1.0 | 1.7 | 1.3 |
| | | -Lactobacillus | 10 ⁶ /g | 226 ± 97 | 37 ± 12 | 164 ± 84 |
| | | | Index | 1.0 | 0.2 | 0.7 |
| | | -Bifidobacterium | 10 ⁶ /g | 500 ± 205 | 197 ± 94 | 759 ± 223 |
| | | | Index | 1.0 | 0.4 | 1.5 |
| | | Probiotics | 10 ⁶ /g | 946 | 608 | 1208 |
| | | | Index | 1.0 | 0.4 | 1.5 |
| | | Probiotics | Ratio% | 8.9 | 7.7 | 2.7 |
| | | | 10 ⁶ /g | 4360 ± 2958 | 38 278 ± 13260 | 29 785 ± 12945 |
| | | | Index | 1.0 | 8.8 | 6.8 |
| | | Probiotic Biofir® (Experimental) | 57 | Total germ number | 10 ⁶ /g | 134 ± 83 |
| Index | 1.0 | | | | 8.8 | 6.8 |
| -Streptococcus | 10 ⁶ /g | | | 134 ± 83 | 593 ± 94 | 930 ± 154 |
| | Index | | | 1.0 | 8.8 | 6.8 |
| -Lactobacillus | 10 ⁶ /g | | | 134 ± 83 | 593 ± 94 | 930 ± 154 |
| | Index | | | 1.0 | 8.8 | 6.8 |
| -Bifidobacterium | 10 ⁶ /g | | | 134 ± 83 | 593 ± 94 | 930 ± 154 |
| | Index | | | 1.0 | 8.8 | 6.8 |
| Probiotics | Index | | | 1.0 | 7.9 | 38.7 |
| | Ratio% | | | 12.7 | 11.5 | 72.0 |

of Lactobacilli showed no change. No significant change was found in faeces pH values in both groups.

The participants followed the prescribed diet but consumed no probiotic food for another two weeks following the 4-wk period of consumption. Microflora analysis of the faeces was repeated in the 6th wk. The results indicated that both the total number of germs and the number of probiotic germs returned to the initial values in both groups.

DISCUSSION

Various types of medical treatment may damage the probiotic flora in human organ. Besides, similar damage can result from the lack of appropriate nutrients in the normal flora. The findings of several controlled studies in the field have proved that various probiotic strains are capable of shortening the duration of gastroenteritis induced by rotavirus. The beneficial effects of probiotics on chronic inflammatory intestinal conditions have been reported. These effects may be attributed to the proteolytic activity of the intestinal flora, which contributes to the breaking up of enteral antigens, reduces secretion of inflammation mediators, helps the normal activity of the intestinal mucosal barrier, normalizes intestinal permeability and increases the production of mucosal IgA^[19,21]. The production of specific IgE, which prevents the allergic reactions, is inhibited by the healthy intestinal microflora^[19,22].

A well-known probiotic effect is that some bacteria produce beta galactosidase, which makes their use desirable and beneficial in the case of lactose intolerance. The decreased activity of faecal enzymes and the low levels of

faecal toxins are due to the healthy intestinal flora, which is an important factor for the prevention of large intestine cancer.

Prebiotics play an important part in the maintenance and regeneration of healthy intestinal flora as they are components of several plant foods and oligosaccharides with beneficial effect on the host organisms by selectively increasing the multiplication of probiotic bacteria and their activity in the large intestine.

One of the probiotic strains of bacteria used in the present study is capable of producing mucus. The mucus produced by it is a polysaccharide, which serves as a prebiotic agent for other probiotic strains. Besides, it increases the preservability of food products containing it and prevents their acidification.

The present study proved that Biofir[®], a probiotic kefir, was capable of promoting the multiplication of the probiotic flora in the large intestine but the traditional Russian type kefir could not. As a result of the consumption of Biofir[®] most probiotic microbes grew significantly but the consumption of traditional kefir did not change the number (index) of these microbes.

In the present study, the initial heterogeneous color and the consistency of faecal samples were totally homogeneous after four weeks of Biofir[®] consumption, probably due to the advantageous bio-physiological processes caused by the multiplication and ultimate predominance of the useful probiotic microbes.

In conclusion, Biofir[®] meets all the criteria of functional foods and can be used in the prevention and treatment of various acute and chronic inflammatory conditions and nutritional allergies. Further investigations should be

undertaken to determine the effect of probiotic strains on various pathological conditions.

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***In vitro* porcine brain tubulin assembly inhibition by water extract from a Chinese medicinal herb, *Tripterygium hypoglaucum* Hutch**

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Abstract

AIM: To investigate the effect of *Tripterygium hypoglaucum* Hutch (THH) on the assembly and disassembly process of tubulin and its possible mode of action.

METHODS: *In vitro* porcine brain tubulin assembly assay was employed to analyze the inhibitory effects of THH at different concentrations (0.05 µg/L, 0.07 µg/L, 0.09 µg/L). Colchicine (0.0025 mmol/L, 0.0050 mmol/L, 0.0075 mmol/L) was used as a positive control.

RESULTS: THH could significantly inhibit the assembly of isolated porcine brain tubulin at all tested concentrations.

CONCLUSION: THH is capable of inducing aneuploidy in mammals via tubulin polymerization inhibition pathway and may pose a genetic risk to human beings.

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Key words: *Tripterygium hypoglaucum* (level) Hutch; Tubulin assembly-disassembly

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INTRODUCTION

Aneuploidy is an important genetic change associated with birth defects, infertility, spontaneous abortions and cancer development [1], which are dependent upon the functioning of a variety of cell organelles and/or a number of division-specific metabolic activities, such as synthesis and functioning of the proteins of the nuclear spindle, attachment and movement of chromosomes on the spindle apparatus. A variety of chemicals are capable of modifying certain functions of spindle apparatus components which may lead to chromosome mal-segregation [2]. The frequency of aneuploidy can be increased by chemical treatment in various experimental cells. If human beings are exposed to aneugens, the risk of developing aneuploidy is increased, which is a matter of concern. Therefore, it is necessary to analyze the aneugenic potential of chemicals in safety assessment since chemically-induced aneuploidy in human beings can not be excluded.

Cochicine and vinblastine are known aneugens which affect tubulin polymerization and depolymerization, and disrupt microtubule formation during cell cycle [3,4]. As tubulin is the main constituent of the spindle, the balance between microtubule assembly and disassembly is critical for the spindle function. *In vitro* tubulin assembly assay is a useful screening system which could give information about the possible functional mechanism(s) of tested chemicals and their potential to cause aneuploidy.

The study of compounds and functions of plant origin has generated a great interest in the fields of food and medicine. Many of them have chemo-preventive properties which need to be tested for their effects on human beings. THH is a traditional Chinese medicinal herb belonging to the genus Celastraceae. The water extract from THH contains alkaloids, dulcitol, terpenes, lactones and pigments. The dry root can be immediately used in the form of decoction or the unpurified water extract can be compacted to tablets for clinical use. THH has been widely used in the treatment of various human immunological diseases, such as systemic lupus erythematosus, rheumatoid arthritis, and others. It was reported that the use of THH can lead to reversible inhibition of germinal cell development both in human beings and in rats. Previous research showed that THH is a potent aneugen [5]. THH induces a significantly higher number of kinetochores

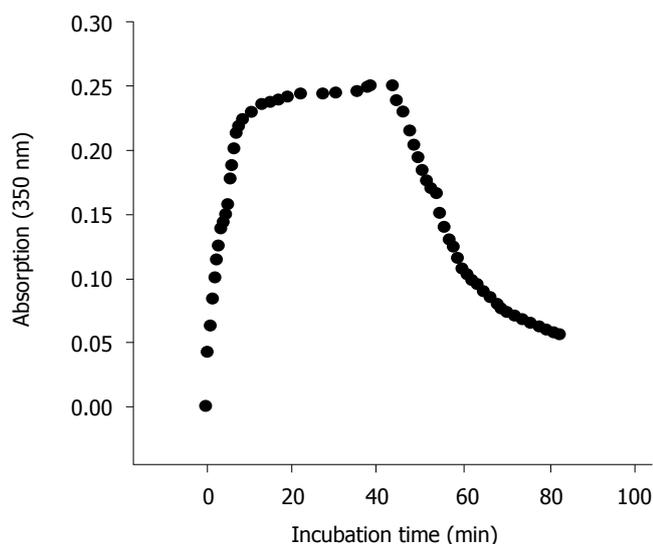


Figure 1 Scheme of *in vitro* tubulin assay. The tubulin assembly was achieved at the end of the absorption after 20 min incubation and kept a steady state level at 37 °C. The disassembly was carried out at 4 °C and after the assembly (40-min incubation).

positive micronuclei both in mouse bone marrow and in mouse NIH 3T3 cells [6, 7]. Recently, we also found that THH could induce aneuploidy of chromosome 8 both in mouse bone marrow cells and in sperm cells by fluorescence *in situ* hybridization (FISH) [8]. The aim of the present study was to investigate the effect(s) of THH on tubulin assembly and disassembly and its possible mode of action. The study may help us to understand the potential adverse effects of THH on human health.

MATERIALS AND METHODS

Chemicals

Dithioerythritol, PIPES, and colchicines (COL) were purchased from Sigma. Guanosine-5'-triphosphate (GTP; Sigma) was a gift from Dr. ID Adler, Germany. Glycerol, MgSO₄ dry herb of THH were purchased from Shanghai Chemical Company, and Kunming Medicine Company, China, respectively.

All the chemicals and THH were freshly prepared. COL was dissolved in sterile distilled water according to the test concentrations (0.0025 mmol/L, 0.0050 mmol/L, 0.0075 mmol/L), and 100g of mashed THH sample was extracted in 1000mL boiling water for 5 min and cooled down to room temperature for 30 min. The procedure was repeated three times to ensure maximum extraction. The supernatant was filtered and lyophilized. The yield of the crude THH extract was about 4.8%. THH extract was dissolved again in sterile distilled water before use according to the test concentrations (0.05 µg/L, 0.07 µg/L, 0.09 µg/L). The chemical and compound concentrations selected in the present study were selected in accordance to our preliminary mitotic block study in V79 cells and their solubility.

Purification of tubulin

The brains were obtained from 3 pigs within 2 h after

sacrifice. Purified tubulin was obtained from porcine brain tissue by two cycles of assembly-disassembly procedure following the method of Williams and Lee [9]. The protein concentrations of the final preparation were determined by the method of Bradford with bovine serum albumin as standard. The resultant tubulin concentration was 3.34-3.75 µg/L. SDS-PAGE was performed to determine the purity of each tubulin preparation. Bovine serum albumin (66.2 Ku) and rabbit actin (43 Ku) were used as standard markers when the average MW of tubulin was 52.5 Ku.

Tubulin assembly-disassembly assays

Tubulin assembly was assayed photometrically by measuring the increase in the absorption at 350 nm (OD_{350nm}). Six hundred µL PM-4M buffer containing 0.1mol/L PIPES, 2 mmol/L EGTA, 1 mmol/L MgSO₄, 4 mol/L glycerol, 2 mmol/L dithioerythritol, pH6.9, as well as 30µL of 50 mmol/L GTP and 1400 µL tubulin solution were mixed thoroughly and poured immediately into a cuvette placed in the temperature-controlled compartment (37°C) of a recording spectrophotometer. The changes of OD_{350nm} were recorded at an interval of 1 min. The absorption remained at the steady state level at the end of the assembly. The temperature of temperature-controlled compartment was decreased to 4°C in 2 min to start the disassembling of tubulin. The OD_{350nm} was again recorded (Figure 1).

Treatment and analysis

In vitro assembly analysis of porcine brain tubulin was carried out by adding 5 µL of THH (0.05 µg/L, 0.07 µg/L, 0.09 µg/L) or positive control (COL, 0.0025 mmol/L, 0.0050 mmol/L, 0.0075 mmol/L) into a ice-both cuvette containing 600µL PM-4M buffer (50, 70, 100 µg/mL), 30 µL of 50 mmol/L GTP and 1400 µL of tubulin solution. The contents were mixed thoroughly and placed immediately in the temperature-controlled compartment of a recording spectrophotometer (37°C). The net OD_{350nm} was recorded at an interval of 1 min. The highest concentration of test compound was selected in accordance with previous laboratory results obtained by *in vitro* mitotic block assay with V79 cells. The solubility was considered as well.

RESULTS

The results of porcine brain tubulin assembly assay with COL and THH are shown in Figure 2. COL led to a very strong inhibition of the *in vitro* polymerization of tubulin at all concentrations. THH resulted in a dose-dependent inhibition of polymerization. However, the potency of assembly inhibition by THH was weaker than COL at all concentrations.

DISCUSSION

Aneuploidy plays a crucial role in the initiation and progression of tumorigenesis as well as in inherited disorders and fetal wastages. Chromosome segregation is dependent on different cell organelles, which is

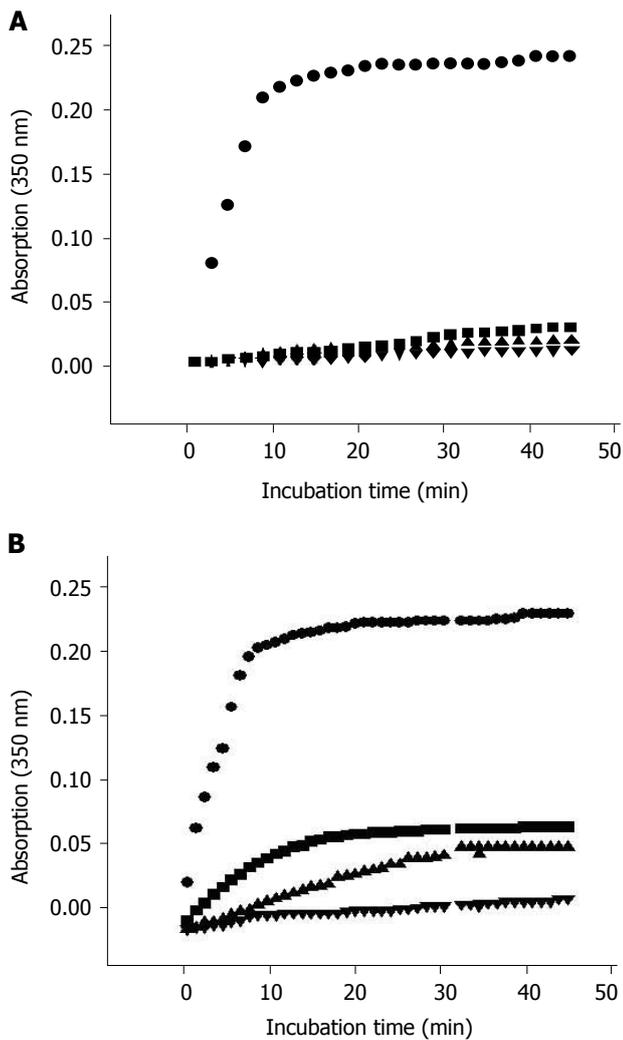


Figure 2 Inhibition of *in vitro* tubulin assembly by COL at the concentration of ■:0.0025 mmol/L, ▲:0.0050 mmol/L, ▼:0.0075mmol/L (A) and THH at the concentration of ■:0.05mg/mL, ▲:0.07g/mL, ▼:0.09mg/mL (B). ●: Solvent control

controlled by a number of metabolic pathways. However, the mechanisms and the cellular targets leading to non-disjunction are largely different from those leading to gene and chromosomal mutations where DNA is the principle target. Aneuploidy-inducing factors include damage to essential elements required for proper chromosome function(s), reduction in chromosomal pairing, induction of chromosome interchange, effect on chromosome condensation, persistence of nucleoli in mitosis or meiosis, increased chromosome stickiness, damage to centrioles or kinetochores, impairment of chromosome alignment, alterations in ion concentrations during mitosis, damage to nuclear membrane, and physical disruption of chromosome segregation [1]. A number of methods have been described for the detection of chemically-induced aneuploidy. However, none of these methods is sufficiently validated for routine screening. Although no conclusive evidence for aneuploidy induction can be obtained from tubulin assembly and disassembly assay, *in vitro* tubulin

assay can be used to pinpoint the aneugenic potential of chemicals by inhibiting the microtubule assembly.

THH has been used to treat many immunological diseases. Both human somatic cells and germ cells are exposed to THH itself or its metabolites during long term clinical use. Since its administration leads to reversible inhibition of germinal cell development both in humans and in rats, there is a great need to evaluate the genetic safety of THH. Our research has shown that THH is a mammalian aneugen both in somatic cells and in germinal cells [5-8]. The results in the present study are in accordance with our previous results, suggesting that either THH itself or its components are capable of inducing aneuploidy via interaction with spindle proteins such as tubulin and probably actin or myosin(s) contained in the contractile ring during cell division. THH may pose a genetic risk to human beings. It seems important for Chinese medical professionals to consider a possible genetic risk of THH and cautiously advise patients to abstain from reproduction within 6 months after the treatment with THH. Further studies are needed to identify the aneugen from THH and to study its possible mode of action.

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RAPID COMMUNICATION

Mutation of RET proto-oncogene in Hirschsprung's disease and intestinal neuronal dysplasia

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Abstract

AIM: To investigate the genetic relationship between Hirschsprung's disease (HD) and intestinal neuronal dysplasia (IND) in Chinese population.

METHODS: Peripheral blood samples were obtained from 30 HD patients, 20 IND patients, 18 HD/IND combined patients and 20 normal individuals as control. Genomic DNA was extracted according to standard procedure. Exons 11,13,15,17 of RET proto-oncogene were amplified by polymerase chain reaction (PCR). The mutations of RET proto-oncogene were analyzed by single strand conformational polymorphism (SSCP) and sequencing of the positive amplified products was performed.

RESULTS: Eight germline sequence variants were detected. In HD patients, 2 missense mutations in exon 11 at nucleotide 15165 G→A (G667S), 2 frameshift mutations in exon 13 at nucleotide 18974 (18974insG), 1 missense mutation in exon 13 at nucleotide 18919 A→G (K756E) and 1 silent mutation in exon 15 at nucleotide 20692 G→A(Q916Q) were detected. In HD/IND combined patients, 1 missense mutation in exon 11 at nucleotide 15165 G→A and 1 silent mutation in exon 13 at nucleotide 18888 T→G (L745L) were detected. No mutation was found in IND patients and controls.

CONCLUSION: Mutation of RET proto-oncogene is involved in the etiopathogenesis of HD. The frequency of RET proto-oncogene mutation is quite different between IND and HD in Chinese population. IND is a distinct clinical entity genetically different from HD.

INTRODUCTION

Hirschsprung's disease (HD) is a frequent congenital malformation affecting one in 5000 births. It is characterized by the absence of ganglion cells in both the myenteric and submucosal plexuses along variable lengths of the hindgut leading to intestinal obstruction in neonates and severe constipation in infants and adults. The occurrence of most HD cases is sporadic, but 15-20% are in familial form^[1]. Genetic analyses have illustrated several genes coding for components of signaling pathways including RET^[2-9], GDNF^[10-12], EDN3^[10,13-15] and EDNBRB^[10,15-18] involved in occurrence of HD. Mutations of RET gene are responsible for a dominant form of HD and account for half of the familial cases and 0-50% of sporadic cases^[4,6,7].

Intestinal neuronal dysplasia (IND), first described in 1971 by Meier-Ruge^[20], is a malformation of the enteric nervous system and shows the clinical features similar to those of HD. The incidence of IND ranges 5-60% of all patients biopsied for suspected HD. The prominent histological features of IND are hyperganglionosis of submucosal and myenteric plexuses, giant ganglia containing more than seven nerve and ectopic ganglion cells^[19,20]. IND often occurs proximal to the aganglionic segment in HD^[19] and less frequently as an isolated condition. The etiology of IND is still unknown. Since IND and HD often occur in combination, the genesis gene of HD may also be involved in occurrence of IND. To clarify the genetic relationship between HD and IND in Chinese population, we performed genetic analysis of exons 11, 13, 15, 17 of RET proto-oncogene in 30 HD patients, 20 IND patients, 18 HD/IND combined patients and 20 normal individuals as controls.

MATERIALS AND METHODS

Case selection and extraction of DNA

The diagnosis of all patients was confirmed histologically

Table 1 Mutations of RET proto-oncogene in HD and IND patients

| Case | Group | Exon | Nucleotide change | Amino acid change | Mutation type |
|------|--------|------|-------------------|-------------------|---------------------|
| 1 | HD | 11 | G15165→A | G667S | Missense mutation |
| 2 | HD | 11 | G15165→A | G667S | Missense mutation |
| 3 | HD | 13 | 18974insG | --- | Frameshift mutation |
| 4 | HD | 13 | 18974insG | --- | Frameshift mutation |
| 5 | HD | 13 | A18919→G | K756E | Missense mutation |
| 6 | HD | 15 | G20692→A | Q916Q | Silent mutation |
| 7 | HD/IND | 11 | G15165→A | G667S | Missense mutation |
| 8 | HD/IND | 13 | T18888→G | L745L | Silent mutation |

by rectal biopsy and resected bowel specimens. The criteria for HD and IND were based on the guidelines put out by Holschneider *et al*^[19] and Merier-Ruge *et al*^[20]. Peripheral blood samples were obtained from all the patients and controls. All samples were anti-coagulated with sodium citrate. After white blood cells were isolated from each blood sample, genomic DNA was extracted according to standard procedure.

Polymerase chain reaction

Two hundred nanograms of genomic DNA was amplified in 50 µL reaction containing 10 mmol/L Tris-HCL (pH8.4), 50mmol/L KCL, 1.5 mmmol/L MgCl₂, 0.5 µmol/L each of two fragment specific primers, 100 µmol/L each of dATP, dGTP, dCTP and dTTP, 2 units of Tap DNA polymerase. The primers were synthesized by Shanghai Shengong Biology Company. Exon11 (forward): 5'-ACACCACCCACCCACAGAT-3'; (reverse):5'-AAGCTTGAAGGCATCCACGG-3' (273bp). Exon13 (forward):5'-GACCTGGTATGGTCATGGA-3'; (reverse):5'-AAGAGGGAGAACAGGGCTGTA-3' (253bp). Exon15 (forward):5'-GACTCGTGCTATTTTCCTAC-3'; (reverse):5'-TATCTTTCCTAGGCTTCCC-3'(234bp). Exon17(forward): 5'-CCCCACTAGATGTATAAGGG-3'; (reverse): 5'-TCACTGGTCTTTCACTCTCT-3' (232bp). After denaturation at 94°C for 5 min, 30 cycles of PCR amplification were carried out at 94°C for 50 s, at 58°C - 62°C for 50 s, at 72°C for 50s, and a final extension at 72°C for 10 min.

SSCP and sequence analysis

The amplified fragments of RET proto-oncogene were analyzed for mutations by SSCP performed on a Mini Electrophoresis Unit (Bio-Rad Company, USA). Ten microliters of PCR products was mixed with 10 µL of loading buffer (90% formamide, 0.05% bromphenol blue dye and 0.05% xylene cyanol), heat denatured at 100°C for 8 min, immediately placed on ice for 3 min, and analyzed by 8% PAGE in 45 Mm-Tris-borate (pH8.0)/1Mm-EDTA (TBE) buffer under 13 v/cm at 10°C. After the gels were stained with silver, the bands were analyzed and photographed. Abnormal PCR products screened by SSCP were purified by VIOGENE kit and sequenced using PE377 automated sequencer.

RESULTS

To determine the mutation of RET proto-oncogene and genomic relationship between HD and IND in Chinese population, we performed PCR-SSCP and sequence

analysis of RET gene mutation in exons 11,13,15,17 in 30 HD patients, 20 IND patients, 18 HD/IND combined patients and 20 normal controls. Eight germline sequence variants were detected. In HD patients, 2 missense mutations in exon 11 at nucleotide 15165 G→A resulting in a G667S exchange in codon 667 and 2 frameshift mutations in exon 13 at nucleotide 18974 (18974insG), 1 missense mutation in exon13 at nucleotide 18919, and 1 silent mutation in exon 15 at nucleotide 20692 (Q916Q) were detected. In HD/IND combined patients, 1 missense mutation in exon 11 at nucleotide 15165 G→A and 1 silent mutation in exon 13 at nucleotide 18888 T→G (L745L) were found. All these mutations were heterozygous (Table 1). No mutation found in IND patients and controls.

DISCUSSION

The RET proto-oncogene is first detected by transfection of NIH 3T3 cells with human lymphoma DNA^[21] which lies on chromosome band 10q11.2 and comprises 20 exons^[22,23]. The product of the RET proto-oncogene is a RET protein, a member of the receptor tyrosine kinase superfamily consisting of a cadherin-like ligand-binding extracellular domain, a transmembrane domain, and an intracellular tyrosine kinase domain^[24]. Genetic studies have demonstrated that the germline mutations of RET gene cause endocrine neoplasia and familial medullary thyroid carcinoma^[25,26]. It was reported that the RET proto-oncogene is expressed in the early stage of embryogenesis and plays an important role in the differentiation of peripheral nervous system and the excretory system^[27,28]. A number of studies showed that RET proto-oncogene is the causative gene for the development HD^[2-11].

IND as a cause of severe chronic constipation remains controversial and shows the clinical features similar to those of HD. Both IND and HD demonstrate hyperplasia of mast cells (MC) and decreased synaptophysin (SY) activity in abnormal bowel^[29,30]. Histological features of IND are hyperganglionosis of submucosal and myenteric plexuses, giant ganglia containing more than seven nerve and ectopic ganglion cells while HD is characterized by the absence of ganglion cells in myenteric and submucosal plexuses. Cajal hyperplasia has been found in IND not in HD^[31,32]. In contrast to the aganglionic segments, the mucin composition of the IND-B segments is normal^[29]. The etiology of IND is still unknown. Kobayashi *et al*^[33] reported that patients with IND have defective innervation at the neuromuscular junction of the affected bowel^[33]. However, IND is not a neuromuscular junction disorder^[34]. Wheatley *et al*^[35] reported that

IND is correlated with deficiency of substance P (SP) in myenteric axons. Although the occurrence of most INDs is sporadic, IND may have dominant autosomal inheritance^[36,37]. Since IND shows the clinical features and certain immunohistochemistry activity similar to those of HD, mutations of RET proto-oncogene, a major causative gene for HD, may also cause IND.

In this study, 6 RET proto-oncogene mutations were detected in 30 HD patients including 1 silent mutation, 3 missense mutations and 2 frameshift mutations. The frequency of RET proto-oncogene mutation was 20% in Chinese HD patients, which is consistent with the reported frequency ranging 0-50%^[4,6,7]. These mutations are heterozygous, suggesting that 50% of the RET proto-oncogene is likely to cause HD^[38].

Two RET proto-oncogene mutations (1 silent mutation and 1 missense mutation) were detected in 18 HD/IND combined patients. However, we could not confirm that the mutation of RET proto-oncogene in HD/IND combined patients can cause IND or HD or both. No RET proto-oncogene mutation was detected in 20 IND patients and 20 normal controls. Our data confirm that the frequency of RET gene mutation is quite different between IND and HD in Chinese population. Gath *et al*^[10] reported that 3 RET proto-oncogene mutations have been detected in 29 HD patients and no RET proto-oncogene mutation has been found in IND or IND/HD patients^[10]. Barone *et al*^[39] performed linkage analysis in two IND pedigrees and demonstrated that IND is not linked to RET proto-oncogene mutation.

In conclusion, IND is a distinct clinical entity genetically different from HD. Further investigation is necessary to elucidate the pathogenesis of IND.

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RAPID COMMUNICATION

Inhibitory effect of antisense vascular endothelial growth factor RNA on the profile of hepatocellular carcinoma cell line *in vitro* and *in vivo*

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effect on cell proliferation and apoptosis of SMMC-7721 *in vitro* but can inhibit tumor growth and induce cell apoptosis *in vivo*.

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Key words: Antisense RNA; Vascular endothelial growth factor; Gene expression; Hepatocellular carcinoma; Transfection

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Abstract

AIM: To evaluate the effect of antisense vascular endothelial growth factor (VEGF) RNA (PCMV-FGEV) transfection on the profile of hepatocellular carcinoma (HCC) SMMC-7721 cells *in vitro* and *in vivo*.

METHODS: SMMC-7721 cells were transfected with PCMV-FGEV antisense, PCMV-VEGF sense and empty vector plasmid encapsulated by lipofectamine as antisense group, sense group and control group respectively. The positive cell clones were selected with G418. The stable transfection and expression of VEGF in the cells were determined by RT-PCR and immunohistochemistry. Cell proliferation was observed by MTT assay. FACS analysis was used to determine the effect of PCMV-FGEV transfection on cell apoptosis. The growth of transfected cells *in vivo* was also observed in nude mice.

RESULTS: VEGF expression was reduced in SMMC-7721 transfected with PCMV-FGEV, which was confirmed by RT-PCR and immunohistochemistry. No effect of PCMV-FGEV transfection was found on cell proliferation and cell apoptosis of SMMC-7721 *in vitro*. The growth of cells transfected with PCMV-FGEV was slow in nude mice and accompanied with obvious apoptosis. The latent time of tumors in the antisense group was 25.0 ± 1.8 d, which was longer than that in sense and control groups ($F = 19.455, P < 0.01$). The average tumor weight in antisense group ($0.96 \text{ g} \pm 0.28 \text{ g}$) was the smallest among the three groups ($F = 21.501, P < 0.01$).

CONCLUSION: The expression of VEGF can be inhibited by antisense PCMV-FGEV. Antisense PCMV-FGEV has no

INTRODUCTION

Angiogenesis plays an essential role in the proliferation and metastasis of tumor cells by supplying them with nutrition and oxygen and disposing waste products. Vascular endothelial growth factor (VEGF) is an important element in angiogenesis and permeability in normal and pathological tissue^[1-6]. Hepatocellular carcinoma (HCC) with an extremely poor prognosis is known to have abundant blood supply. VEGF has been reported to play an important role in the angiogenesis of HCC^[7-11]. Due to the high expression level of VEGF mRNA in HCC, antisense RNA is used to elucidate the possible therapeutic effects on HCC. Using this experimental approach, we explored the effects of antisense PCMV-FGEV transfection on the profile of HCC SMMC-7721 cells *in vitro* and *in vivo*.

MATERIALS AND METHODS

Reagents

TRIzol, RPMI1640 medium, 10% fetal bovine serum (FBS), lipofectamine, G418 were purchased from GIBCO (Carlsbad, CA, USA). VEGF and factor VIII antibody were purchased from Boster (Wuhan, China). PI and MTT were purchased from Sigma (St. Louis, MO, USA). VEGF sense, antisense vector PCMV-VEGF, PCMV-FGEV and empty vector PcDNA3.1 were given as gifts by NIH, USA.

Experimental animals

Twelve female athymic BALB/c-nu/nu nude mice at the age of 4-6 wk were purchased from Chinese Academy of Sciences and maintained under conditions that met all requirements for use in an approved facility.

Cell culture

Human HCC SMMC-7721 cells were obtained from the Central Laboratory of Tianjin Cancer Hospital and cultured in RPMI1640 medium containing 100 U/mL penicillin G sodium and 100 U/mL streptomycin sulfate, supplemented with 10% fetal calf serum at 37°C in a 50mL/L CO₂ atmosphere.

Transfection

Cells were transfected with antisense PCMV-FGEV (antisense group), sense PCMV-VEGF (sense group) and empty vector PcDNA3.1 (control group) encapsulated by lipofectamine. Forty-eight hours after transfection, cells were diluted and plated into tissue culture dishes for 4 wk in complete growth medium containing 400 µg/mL G418. Colonies resistant to G418 were isolated.

Immunohistochemistry

Immunohistochemical analysis was performed on formalin-fixed, paraffin-embedded sections using a three-step indirect method for VEGF and factor VIII expression.

RT-PCR

Guanidinium isothiocyanate-phenol single-step method was used to extract total RNA from cells using TRIzol following instructions of the kit. The integrity, purity and concentration of the extracted mRNA were detected with ultraviolet spectral photometer and agarose gel electrophoresis. The RNA extract dissolved in DEPC was stored at -70°C. According to the sequences of human mRNA of VEGF, the primers were designed, synthesized and supplied by Boya Biotech, Shanghai, China (VEGF positive primer: 5'-AATGCTTTCTCCGCTCTG-3', negative primer: 5'-TTGCTGCTCTACCTCCAC-3'; β-actin positive primer: 5'-TTGCGCTCAGGAGGAGC AAT-3', negative primer: 5'-TTCCAGCCTTCCTTCCTG G-3'). First-strand cDNA was synthesized from 2µg RNA dissolved in water administered with DEPC, in which 0.2 µL oligo dT was added at 70°C for 5 min and placed on ice for 1 min, followed by 1µL M-MLV reverse transcriptase (200IU/µL), 0.5µL RNasin (40 IU/µL), 8µL 5×RT buffer, and 3µL dNTPs (10 mmol/L) at 42°C for 60 min. The reverse transcriptase was inactivated at 95°C for 5 min, then 5µL cDNA, 5µL 10×buffer, 3µL 2mmol/L dNTPs, 1µL 5 IU/L LA Taq enzyme and water were added to a volume of 40µL. The final concentrations of positive and negative primers were both 1µM/L. VEGF was amplified for 35 cycles at 94°C for 1 min, at 55°C for 1 min, at 72°C for 2 min, and a final extension at 72°C for 7 min. The area of electrophoresis bands (AREA), the absorption of mean optical density (A) and product of AREA and A were quantitatively analyzed after the PCR product was detected with auto image manipulating system following agarose gel (2%) electrophoresis and stained with EB. The samples were controlled with blank and β-actin.

MTT assay

For analysis of the transfected SMMC-7721 cell proliferation, SMMC-7721 cells were seeded in a 96-well plate (6000 cells/well), untransfected SMMC-7721 cells were used as negative control. After further incubated for 24, 48, 72, 96, 120, 144 h, cell proliferation activity was determined by MTT assay. The absorbance of each well was measured at 580nm in a microtiter reader. The survival rate of tumor cells was calculated according to the formula: survival rate (%) = (A/B) × 100, where A is the absorbance of treated cells, and B is the absorbance of negative control cells.

Flow cytometry of apoptosis

To investigate the influence of transfection on apoptosis, cells grown as a monolayer were incubated for 24 h, trypsinized, washed with PBS and fixed with 70% ethanol overnight at 4°C. Then cells were intensively washed three times with PBS and incubated with 1% RNase for 30 min at 37°C. Cells were measured with a FACScan flow cytometer (BD Biosciences) equipped with a 488 nm argon-ion laser and a Macintosh Power PC (G4). In general, 25 000 events were acquired using CellQuest Pro 4.0.1. Apoptotic cells were then calculated in percent using ModFIT Vers. 3.0 (BD Biosciences).

Tumorigenesis in nude mice

Cells were resuspended at the density of 5×10^7 cells in 400 µL of RPMI1640 and injected subcutaneously into the flank region of athymic nude mice. Twelve mice were distributed to antisense group, sense group or negative control group, 4 per group at random. All animals were observed for up to 10 wk following the injection, and then the tumor was excised.

Transmission electron microscopy

For electron microscopy, small blocks of tumor tissue were fixed in 1% glutaraldehyde and 4% paraformaldehyde, postfixed in 1% osmium tetroxide and embedded in Epon 812, double-stained with uranyl acetate and lead citrate, and observed under a transmission electron microscope.

Statistical analysis

One-way analysis of variance (ANOVA) was used to compare the results between the different groups. All data were processed with SPSS10.0 statistical software. $P < 0.05$ was considered statistically significant.

RESULTS

Immunohistochemistry and RT-PCR of SMMC-7721 cells

VEGF protein expression was high in sense group, moderate in control and 7721 groups and weak in antisense group ($F=16.786$, $P<0.01$). The same results were also confirmed by VEGF mRNA expression in RT-PCR analysis (Figure 1). The mRNA expression of VEGF was seen in all groups. However, compared to the control and sense groups, the expression was decreased in the antisense group ($F=19.693$, $P<0.01$), suggesting that SMMC-7721 cells transfected with PCMV-FGEV could effectively inhibit VEGF expression at protein and RNA level.

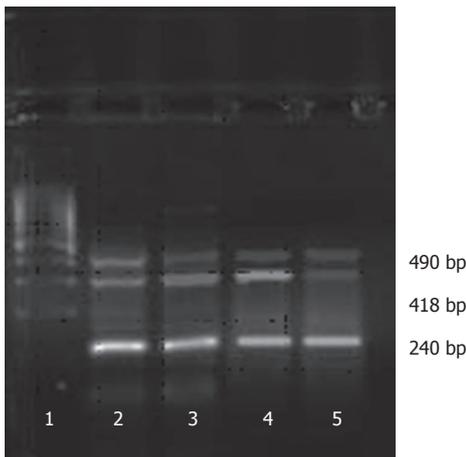


Figure 1 Expression of VEGF mRNAs. Lanes 1- 5: 7721 group ,control group, sense group and antisense group respectively (418 bp and 490 bp indicate VEGF, 240 bp indicates β -actin).

Proliferation and apoptosis of transfected SMMC-7721 cells

We used MTT assay to assess the proliferation of transfected SMMC-7721 cells *in vitro*. The difference in survival rates of the cells in sense group, antisense group, and control group was not significant ($F=0.869$, $P>0.05$). Further we used flow cytometry to compare the extent of cell apoptosis among the different groups and did not find the characteristic changes of cell apoptosis in each group.

Time of tumorigenesis in nude mice and weight of tumors

The time of tumorigenesis was 25.0 ± 1.8 d in antisense group, 15.7 ± 2.5 d in sense group and 18.5 ± 2.1 d in control group ($F=19.445$, $P<0.01$). The weight of tumor was 0.96 ± 0.28 g in antisense group, which was obviously lighter than that in sense group (2.18 ± 0.36 g) and control group (1.88 ± 0.47 g) ($F=21.505$, $P<0.01$)(Figure 2).

Immunohistochemistry for VEGF and VIII factor expression

VEGF immunohistochemical staining showed faint signals in antisense group. The number of positive cells and intensity of staining were significantly lower than those in sense or control group ($F=21.365$, $P<0.01$). Factor VIII staining showed that the number of tumor vessels in antisense group was significantly less than that in sense or control group ($F=9.985$, $P<0.01$).

Tumor apoptosis assay

Electron micrographs of the tumor cells in antisense group showed characteristic chromatin condensation forming a crescentic-like cap, which was characteristic appearance of apoptosis. Comparatively, such chromatin change of the cells was not detectable in control or sense group ($F=24.548$, $P<0.01$).

DISCUSSION

Hepatocellular carcinoma (HCC) is one of the most common malignant neoplasms. All the treatment strategies used today have no good curative effect^[12]. Gene therapy

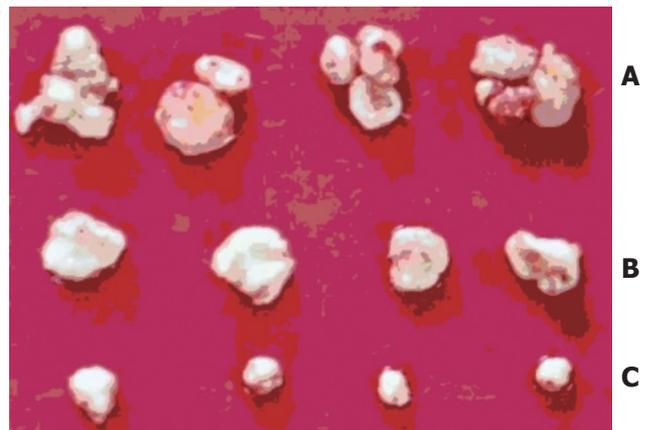


Figure 2 Gross appearance of tumor growth in sense group (A), control group (B) and antisense group (C). The weight of tumor in antisense group was obviously lighter than that in sense and control groups ($F=21.505$, $P<0.01$).

might be a promising way. Gene transfer is a key technique in gene therapy. Antisense gene technologies have been proven to be powerful tools for selective regulation of gene expression in experimental settings and are under evaluation for their therapeutic potential in clinic^[13-16]. Antisense agents down-regulate the expression of specific target genes at mRNA level by pairing with their complementary RNA and preventing their translation into proteins. Theoretically, antisense molecules could be used to cure a variety of diseases, especially some cancers^[17-20].

VEGF plays an important role in the angiogenesis of HCC. VEGF, known as a vascular permeability factor, has two major biological functions: growth stimulatory activity for a variety of vascular endothelial cells and increasing microvascular permeability^[21-23]. Use of antisense VEGF RNA for inhibiting vessel formation in HCC might be a rational approach. Our study showed that antisense VEGF RNA could effectively inhibit the expression of VEGF in HCC cell line SMMC-7721, but could not significantly inhibit growth of the cells *in vitro*, which is in accordance with the report of Gu *et al*^[24]. The reasons may be as follows: VEGF is specific to endothelial cells and there is no vessel in the HCC cells. Although antisense VEGF RNA can inhibit the expression of VEGF, it can not effectively inhibit tumor cells without vessels. Furthermore If we use the vascular endothelial cells as the model to study the inhibitory effect of antisense VEGF RNA on them, the results may be that antisense RNA could inhibit not only the expression of VEGF but also the growth of vascular endothelial cells.

This study also demonstrated that antisense VEGF RNA could inhibit the growth of tumor *in vivo*. The secretion of VEGF and the vessels of tumor in antisense group were significantly decreased, leading to inhibition of tumor growth and metastasis. Becker *et al*^[25] reported that transfecting the gene of soluble vascular endothelial growth factor receptor flk-1 to the prostate cancer nude model can inhibit tumor growth and metastasis. Zhang *et al*^[26] found that angiogenic inhibition mediated by DNase targeting at vascular endothelial growth factor receptor -2 could inhibit the formation of vessels and the growth of tumor.

In conclusion, antisense VEGF RNA has inhibitory effect on the growth of HCC cells *in vitro* and *in vivo* and can be used in the treatment of HCC.

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RAPID COMMUNICATION

DNA damage, apoptosis and cell cycle changes induced by fluoride in rat oral mucosal cells and hepatocytes

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Abstract

AIM: To study the effect of fluoride on oxidative stress, DNA damage and apoptosis as well as cell cycle of rat oral mucosal cells and hepatocytes.

METHODS: Ten male SD rats weighing 80~120 g were randomly divided into control group and fluoride group, 5 animals each group. The animals in fluoride group had free access to deionized water containing 150 mg/L sodium fluoride (NaF). The animals in control group were given distilled water. Four weeks later, the animals were killed. Reactive oxygen species (ROS) in oral mucosa and liver were measured by Fenton reaction, lipid peroxidation product, malondialdehyde (MDA), was detected by thiobarbituric acid (TBA) reaction, reduced glutathione (GSH) was assayed by dithionitrobenzoic acid (DTNB) reaction. DNA damage in oral mucosal cells and hepatocytes was determined by single cell gel (SCG) electrophoresis or comet assay. Apoptosis and cell cycle in oral mucosal cells and hepatocytes were detected by flow cytometry.

RESULTS: The contents of ROS and MDA in oral mucosa and liver tissue of fluoride group were significantly higher than those of control group ($P < 0.01$), but the level of GSH was markedly decreased ($P < 0.01$). The contents of ROS, MDA and GSH were (134.73 ± 12.63) U/mg protein, (1.48 ± 0.13) mmol/mg protein and (76.38 ± 6.71) mmol/mg protein in oral mucosa respectively, and (143.45 ± 11.76) U/mg protein, (1.44 ± 0.12) mmol/mg protein and (78.83 ± 7.72) mmol/mg protein in liver tissue respectively. The DNA damage rate in fluoride group was 50.20% in oral mucosal cells and 44.80% in hepatocytes, higher than those in the control group ($P < 0.01$). The apoptosis rate in oral mucosal cells was (13.63 ± 1.81) % in fluoride group, and (12.76 ± 1.67) % in hepatocytes, higher than those in control group. Excess fluoride could differently lower the number of oral mucosal cells and hepatocytes at G₀/G₁ and S G₂/M phases ($P < 0.05$).

CONCLUSION: Excess fluoride can induce oxidative

stress and DNA damage and lead to apoptosis and cell cycle change in rat oral mucosal cells and hepatocytes.

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Key words: Fluoride; Oxidative stress; DNA damage; Apoptosis; Cell cycle

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INTRODUCTION

Fluoride is an essential trace element for human beings and animals. Fluoride can prevent caries and enamel fluorosis. Caries is the demineralization of the enamel by acids produced by plaque bacteria, leading to cavitation. Enamel fluorosis is a subsurface hypomineralization of the dental enamel caused by chronic ingestion of high fluoride concentration while the dentition is forming^[1]. Other manifestations of fluoride toxic effects include skeletal fluorosis and damage to kidney, liver, parathyroid glands and brain^[2-4]. Lipid peroxidation is implicated as an important mechanism of fluorosis. A close association between fluoride toxicity and oxidative stress has been reported in human beings^[5], experimental animals^[3] and cultured cells^[2]. Studies have shown that excess fluoride can cause DNA damage, trigger apoptosis and change cell cycle^[2,6]. Jeng *et al*^[7] studied the effects of sodium fluoride on human oral mucosal fibroblasts and found that sodium fluoride is toxic to oral mucosal fibroblasts *in vitro* by inhibiting protein synthesis, disturbing mitochondrial function and depleting cellular ATP. The effects of sodium fluoride on cultured human oral keratinocytes have been investigated with respect to induction of unscheduled DNA synthesis. These researches indicate that fluoride has harmful effects on oral mucosa. In the present study, we investigated the effects of sodium fluoride (NaF) on lipid peroxidation, DNA damage and apoptosis in rat oral mucosal cells and hepatocytes.

MATERIALS AND METHODS

Animals

Male SD rats weighing 80-120 g were used in the

experiments and housed in polycarbonated cages with compressed fiber bedding. Commercial pellet diet and water were provided *ad libitum*. The animals were divided into control group and fluoride group, 5 animals each group. The control group was given distilled water and the fluoride group was provided with distilled water containing 150 mg/L sodium fluoride. The animals were sacrificed four weeks later and their oral mucosa and liver were removed immediately for use.

Determination of fluoride concentration in urine and blood

At the end of experiments, the animals were held in plastic metabolic cages for 8 h, and urine was collected in the container. Concentration of fluoride in urine and blood was determined with fluoride ion-selective electrodes as described by Zhang *et al.*^[8].

Preparation of tissue extract

Oral mucosa and liver were minced and homogenized in 50 mmol/L cold sodium phosphate buffer (pH 7.0) containing 0.1 mmol/L EDTA to produce 10% homogenates (W/V). The homogenates were then centrifuged at 1000 r/min for 10 min at 4 °C. The supernatants were separated and used for enzyme assays and protein determination.

Determination of GSH, LPO and ROS

The reduced glutathione (GSH) was determined in tissues by the method of Kum-Talt and Tan^[9] using dithionitrobenzoic acid (DTNB) reagent and the absorbance at 412 nm was measured. Lipid peroxidation products (LPOs) were determined by measuring the levels of malondialdehyde (MDA)^[10]. To 0.2 mL of homogenates 0.2 mL 8.1% (w/v) sodium dodecyl sulphate and 1.5 mL 20% acetic acid were added, and pH was adjusted to 3.2 with 20% (w/v) sodium acetate solution. After the addition of 1.5 mL of thiobarbituric acid (0.8%, w/v) the mixture was diluted to 4 mL with water, heated for 60 min in boiling water bath and cooled to room temperature. Then 1 mL of water and a mixture of n-butanol and pyridine (15:1) were added. The mixture was shaken vigorously and centrifuged at 1500 r/min for 15 min. The absorbance of organic layer was measured at 532 nm and the results were expressed as mmol MDA/mg protein. Reactive oxygen species (ROS) was measured with test kit (Nanjing Jiancheng Biological Technology Company) by Fenton reaction. Gress reagent was used to initiate color reaction and the absorbance was read at 510 nm on a spectrophotometer. Protein content was determined by the method of Lowry using bovine serum albumin as a reference^[11].

Detection of DNA damage

Single cell gel electrophoresis assay (also known as comet assay) was performed as previously described^[12] with some modifications. Approximately 1.0×10^5 cells were embedded in low melting agarose (0.65%) that was layered onto fully frosted microscope slides coated with a layer of 0.75% normal agarose (diluted in Ca²⁺ and Mg²⁺ free PBS buffer). A final layer of 0.65% low-melting agarose was placed on top. Slides were immersed in a jar containing cold lysate solution (1% Triton X-100, 10% DMSO and 89% of 10 mmol/L Tris, 1% sodium laurylsarcosine, 2.5 mol/L NaCl,

100 mmol/L Na₂EDTA, pH 10) at 4 °C for 1-2 h. Then, slides were pretreated for 15 min in electrophoresis buffer (300 mmol/L NaOH/1 mmol/L Na₂EDTA, pH 12) and exposed to 25 V/300 mA for 20 min. Pre-incubation and electrophoresis were performed in ice bath. Slides were neutralized for 3 - 5 min in 0.4 M Tris, pH 7.5 and DNA was stained by adding 50 µL of ethidium bromide (20 µg/mL) onto each slide. After staining for 5 min, slides were rinsed in distilled water and covered again for microscopic examination. All steps were conducted under red light to prevent additional DNA damage.

Image analysis was performed with 200 × magnification using a fluorescence microscope (Olympus B-60F5) equipped with an excitation filter of 549 nm and a 590 nm barrier filter, coupled to a CCD camera (Kodak, USA). One hundred randomly selected cells per slide were scored. In this test, DNA damage of the cells was evaluated using the ratio of tail DNA content/the whole cellular DNA content.

Detection of apoptosis

DNA fragmentation during apoptosis could lead to extensive loss of DNA content and a distinct sub-G₁ peak when analyzed by flow cytometry. Apoptosis was analyzed by the determination of sub-G₁ cells. At the end of designated treatments (such as cell separation), cells were washed, fixed and permeated with 70% ice-cold ethanol at 4 °C for 2 h. Cells were then incubated with freshly prepared propidium iodide (PI) staining buffer (0.1% Triton X-100, 200 µg/mL RNase A, and 20 µg/mL PI in PBS) at 37 °C for 15 min, followed by flow cytometry of 20 000 cells from each animal. The histogram was abstracted and percentage of cells in the sub-G₁ phase was then calculated to reflect the percentage of apoptotic cells. In addition, cell cycle was analyzed with ModFit LT software.

Statistical analysis

The data were tested with statistical programs. Student's *t* test or Chi-square test was used. *P* < 0.05 was considered statistically significant.

RESULTS

Amount of fluoride in urine and concentration of fluoride in blood

Animals in fluoride group were provided with distilled water containing 150 mg/L NaF for four weeks. The amount of fluoride in urine and concentration of fluoride in blood were higher than those in control group (*P* < 0.01), demonstrating that animals in fluoride group were in excess fluoride status (Table 1).

Content of ROS, MDA and GSH in oral mucosa and liver

ROS and MDA contents in oral mucosa and liver were higher in fluoride group than in control group, but GSH content in oral mucosa and liver was lower in fluoride group than in control group, indicating that oxidative stress was induced in fluoride group (Table 2).

DNA damage in oral mucosal cells and hepatocytes

Table 3 shows the effects of fluoride at the dose of 150

Table 1 Amount of fluoride in urine and concentration of fluoride in blood (mean ± SD)

| Groups | Fluoride in urine (μg) | Concentration of fluoride in blood (mg/L) |
|----------|---------------------------|---|
| Control | 14.22 ± 1.33 | 0.334 ± 0.023 |
| Fluoride | 53.02 ± 5.45 ^b | 1.101 ± 0.123 ^b |

^bP < 0.01 vs control group.**Table 2 Content of ROS, MDA and GSH in oral mucosa and liver (mean ± SD)**

| Groups | ROS (U/mg protein) | MDA (mmol/mg protein) | GSH (mmol/mg protein) |
|------------------------|-----------------------------|--------------------------|---------------------------|
| Control (oral mucosa) | 81.21 ± 7.87 | 0.66 ± 0.05 | 127.50 ± 13.11 |
| Fluoride (oral mucosa) | 134.73 ± 12.63 ^b | 1.48 ± 0.13 ^b | 76.38 ± 6.71 ^b |
| Control (liver) | 75.57 ± 8.05 | 0.71 ± 0.06 | 130.08 ± 12.65 |
| Fluoride (liver) | 143.45 ± 11.76 ^b | 1.44 ± 0.12 ^b | 78.83 ± 7.72 ^b |

^bP < 0.01 vs control group.**Table 3 Effects of fluoride on DNA damage in rat oral mucosal cells and hepatocytes**

| Groups | Cells | Grade of DNA damage | | | | | Rates of comet assay |
|-------------------------------|-------|---------------------|-----|----|----|----|----------------------|
| | | 0 | 1 | 2 | 3 | 4 | |
| Control (oral mucosal cells) | 500 | 448 | 39 | 6 | 5 | 2 | 10.40 |
| Fluoride (oral mucosal cells) | 500 | 249 | 119 | 49 | 54 | 29 | 50.20 ^b |
| Control (hepatocytes) | 500 | 453 | 23 | 12 | 9 | 3 | 9.40 |
| Fluoride (hepatocytes) | 500 | 276 | 127 | 52 | 31 | 14 | 44.80 ^b |

^bP < 0.01 vs control group.**Table 4 Effects of fluoride on apoptosis in rat oral mucosal cells and hepatocytes (mean ± SD)**

| Groups | Oral mucosal cells (%) | Hepatocytes (%) |
|----------|---------------------------|---------------------------|
| Control | 5.61 ± 1.98 | 5.72 ± 1.82 |
| Fluoride | 13.63 ± 1.81 ^b | 12.76 ± 1.67 ^b |

^bP < 0.01 vs control group.**Table 5 Effects of fluoride on proliferation in rat oral mucosal cells and hepatocytes (mean ± SD)**

| Groups | G ₀ /G ₁ (%) | S(%) | G ₂ /M(%) | PI |
|-------------------------------|------------------------------------|--------------|--------------------------|--------------|
| Control (oral mucosal cells) | 62.75 ± 3.89 | 9.28 ± 2.56 | 8.74 ± 1.23 | 21.89 ± 3.61 |
| Fluoride (oral mucosal cells) | 58.53 ± 2.18 | 8.09 ± 1.31 | 6.28 ± 1.25 ^a | 19.51 ± 0.89 |
| Control (hepatocytes) | 64.79 ± 3.91 | 10.28 ± 2.64 | 10.67 ± 1.42 | 22.18 ± 3.59 |
| Fluoride (hepatocytes) | 60.58 ± 2.27 | 9.07 ± 1.42 | 7.98 ± 1.36 ^a | 19.91 ± 1.02 |

^aP < 0.05 vs control group.

mg/L for 4 wk on the grades of DNA damage based on the comet assay. Statistical analysis yielded significant

differences between oral mucosal cells and hepatocytes based on chi-square test ($P < 0.01$). Although the grades of DNA damage in oral mucosal cells were higher than those in hepatocytes, there was no significant difference ($P > 0.05$).

Apoptosis in oral mucosal cells and hepatocytes

Table 4 shows the effects of fluoride at the dose of 150 mg/L for 4 wk on apoptosis in oral mucosal cells and hepatocytes based on flow cytometry. There was a statistically significant difference between fluoride and control groups in apoptotic rate of oral mucosal cells and hepatocytes. Although the apoptotic rate was higher in oral mucosal cells than in hepatocytes, there was no significant difference ($P > 0.05$).

Changes of cell cycle in oral mucosal cells and hepatocytes

Table 5 shows the effects of fluoride on rat cell cycle and proliferation index (PI) of oral mucosal cells and hepatocytes. The number of oral mucosal cells and hepatocytes at G₀/G₁, S and G₂/M phase induced by fluoride was lower than that in control group. PI of oral mucosal cells and hepatocytes was lower in fluoride group than in control group ($P > 0.05$), suggesting that fluoride at the dose of 150 mg/L in drinking water for 4 wk interfered with normal cell cycle in oral mucosa and liver.

DISCUSSION

Fluorosis is one of the diseases caused by biogeochemical factors. Fluorosis in human beings is mainly caused by drinking water, burning coal and drinking tea. China is one of the countries where fluorosis is most endemic^[2]. Fluoride intoxication causes damages to osseous tissue (teeth and bone) and soft tissues (liver, kidney, brain, mucosa, etc.). There are many reports on the mechanisms of skeletal and dental fluorosis^[13, 14], but how fluoride interferes with soft tissue has not been clarified. In our study, male SD rats were provided with distilled water containing 150 mg/L sodium fluoride for four weeks. The concentration of fluoride in blood and the amount of fluoride in urine in fluoride group were significantly higher than those in control group, suggesting that the experimental animals are in excess fluoride status. The content of ROS and MDA was increased, but content of GSH was decreased in oral mucosa and liver, demonstrating that lipid peroxidation can be induced by fluoride intoxication in oral mucosa and liver.

Studies have shown that fluoride can induce excessive production of oxygen free radicals and decrease the biological activities of some substances, such as catalase, superoxide dismutase, xanthine oxidase and glutathione peroxidase, which play an important role in antioxidation and eliminating free radicals. Karaoz *et al*^[15] showed that chronic fluorosis can lead to lipid peroxidation and kidney tissue change in first- and second-generation rats^[15]. Shanthakumari *et al*^[3] showed that the level of lipid peroxides is increased but the activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) are decreased in rats after administered fluoride. Shivarajashan-

kara *et al.*^[16] reported that long-term high-fluoride intake at the early developing stages of life enhances oxidative stress in blood, disturbing the antioxidant defense of rats, suggesting that increased oxidative stress may be one of the mediating factors in the pathogenesis of toxic manifestations of fluoride. Reddy *et al.*^[17] evaluated the antioxidant defense system (both enzymatic and non-enzymatic) and lipid peroxidation both in human beings from an endemic fluorosis area (5 ppm fluoride in drinking water) and in rabbits receiving water containing 150 ppm of fluoride for six months, and found that there is no significant difference in lipid peroxidation, glutathione and vitamin C in blood of fluorosis patients and fluoride-intoxicated rabbits as compared to the controls. They also found that there are not any changes in the activities of catalase, superoxide dismutase, glutathione peroxidase, or glutathione S-transferase in the blood due to fluoride intoxication (of rabbits) or fluorosis in human beings^[17]. Our results are concordant with other studies^[2, 3, 5, 15, 16]. Liver is one of the target organs attacked by excessive amount of fluoride. Evidence of toxic changes in liver has been revealed by long term investigations of industrially-induced fluorosis, including abnormal metabolic functions, reduced activity of detoxication reactions and altered structure of subcellular organelles^[18]. Our results also displayed that excess fluoride could induce oxidative stress not only in liver, but also in rat oral mucosa.

It was reported that DNA damage results from excess fluoride in human embryo hepatocytes^[2, 6] and pallium neurons^[19]. Our results showed that DNA damage was induced in rat oral mucosal cells and hepatocytes by sodium fluoride at the dose of 150 mg/L in drinking water for four weeks. Apoptosis is a programmed physiological process of cell death characterized by a distinct set of morphological and biochemical changes, including cytoplasmic membrane blebbing, apoptotic body formation, nuclear condensation and chromosomal DNA fragmentation. Apoptosis can be triggered in a wide variety of cell lines by diverse stimuli, ranging from extracellular signals to intracellular events^[20]. Previous studies have shown that fluoride induces apoptosis in human embryo hepatocytes^[2, 6], human epithelial lung cells^[21], human and rat pancreatic islets and RINm5F cells^[22] as well as in HL-60 cells^[23]. In the present study, we investigated the effects of fluoride on apoptosis of rat oral mucosal cells and hepatocytes. As regards to the mechanisms of apoptosis induced by fluoride, Wang *et al.*^[2] concluded that fluoride could cause lipid peroxidation, DNA damage and apoptosis, and there is a positive relationship among these changes. Lipid peroxidation and apoptosis may co-exist in the beginning when tissues are exposed to excess fluoride and generates a lot of free radicals that may be sufficient to cause apoptosis. Anuradha *et al.*^[23] showed that sodium fluoride (NaF) induces apoptosis by oxidative stress-induced lipid peroxidation, and thereby releasing cytochrome C into the cytosol and further triggering caspase cascade leading to apoptotic cell death in HL-60 cells. Refsnes *et al.*^[21] reported that NaF induces apoptotic effects and increases PI-positive A549 cells via similar mechanisms, involving protein kinase C (PKC), protein kinase A (PKA), tyrosine kinase and Ca²⁺-linked enzymes. Thrane *et al.*^[24]

found that activation of mitogen-activated protein (MAP) kinases p38 and possibly c-Jun N-terminal kinase (JNK) are involved in NaF-induced apoptosis of epithelial lung cells, whereas extracellular signal regulated kinase (ERK) activation seems to counteract apoptosis in epithelial lung cells. These studies indicate that fluoride induces apoptosis through mechanisms of oxidative stress, caspase and PKC activation, MAPK signal pathway and DNA damage.

Our study also demonstrated that the number of oral mucosal cells and hepatocytes in G₂/M phase was significantly lower in fluoride group than in control group, although there were no obvious changes in cell number in G₀/G₁ and S phase. In our study, proliferation index of oral mucosal cells and hepatocytes was also decreased in fluoride group. Wang *et al.*^[2] showed that the number of human embryo hepatocytes in S phase is significantly higher in fluoride treated groups than in control group, but there were no changes in cell number in G₀/G₁ and G₂/M phase. This may be due to the differences in cell sensitivity to fluoride in different phases of cell cycle^[25].

In the present study, the results in oral mucosal cells were not different from those in hepatocytes significantly. Oxidative stress, DNA damage, apoptosis and modifications of membrane lipids can be induced in hepatocytes by excess fluoride^[2, 3, 6, 18]. It is difficult to get liver tissue but easy to get oral mucosal cells from patients with fluorosis. Squier *et al.*^[26] reported that ethanol exerts its effect on lipid metabolism and epithelial permeability barrier of oral mucosa in rats. Hansson *et al.*^[27] have analyzed proliferation, apoptosis and keratin expression in cultured normal and immortalized human oral mucosal keratinocytes. Dhillon *et al.*^[28] showed that oral mucosa cells have a smaller increase in gamma-ray-induced DNA strand breaks than lymphocytes, suggesting that oral mucosal cells can be used as experimental materials to study oxidative stress, DNA damage and apoptosis as well as other effects of fluoride *in vivo*.

In conclusion, excess fluoride induces oxidative stress, DNA damage, apoptosis and cell cycle changes in rat oral mucosal cells and hepatocytes. Further investigation is needed to clarify the exact mechanisms.

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Primary liposarcoma of esophagus: A case report

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Abstract

Liposarcoma is the most common soft tissue sarcoma in adult life while esophageal liposarcoma is an extremely rare tumor. In the world literature, only 14 cases of esophageal liposarcomas have been described. We report a 72-year old male patient who was urgently admitted to our hospital for acute epigastric pain with a burning retrosternal sensation, persistent nausea, vomiting and dysphagia. Barium swallow, upper gastrointestinal (GI) endoscopy, esophageal manometry and CT scan, failed to accurately diagnose the lesion. After surgical resection of an esophageal polypoid tumor, the histological examination revealed a well-differentiated grade I liposarcoma. Diagnostic and therapeutic tools were discussed and the results of literature were reviewed.

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Key words: Liposarcoma; Esophagus; Surgical treatment

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INTRODUCTION

Liposarcoma is the most common soft tissue sarcoma in

adult life and usually occurs in the retroperitoneum and deep soft tissues of the trunk and lower extremities^[1-3] representing around 20% of mesenchymal neoplasms. It is very rare in the gastrointestinal (GI) tract, exceedingly in the esophagus. Esophageal liposarcomas grow as polypoid masses within the esophageal lumen, usually causing no symptoms until they reach a substantial size when impaired esophageal transit develops. The first case of a primary esophageal liposarcoma was reported by Mansour *et al*^[2] in 1983. The review of the literature revealed another 13 cases that have been reported since. We described a male patient who, to our belief, represents the 15th case reported in the literature, as he was found to have a well-differentiated esophageal liposarcoma arising from the wall of the lower thoracic esophagus exceeding down to the gastroesophageal (GE) junction.

CASE REPORT

A 72-year-old obese male (BMR > 30) was urgently admitted to our hospital because of an acute epigastric pain with a burning retrosternal sensation, persistent nausea, vomiting and complete dysphagia. He suffered from intermittent dysphagia in the last few years which was increasingly worsened in the last 2 mo. The day before his admission to our hospital he had an esophagogram and an esophageal manometry which showed a slightly dilated lower thoracic esophagus without any stricture and an achalasia like motor dysfunction. His past medical history revealed 5 mild episodes of a bleeding duodenal ulcer (from May 1962 to February 1995) which were treated with blood transfusions, antacids and PPIs. He was also under treatment with captopril and furosemide for arterial hypertension in the last ten years.

On initial urgent upper GI endoscopy, a smooth and ulcerated hemorrhagic tumor like protrusion in the lower thoracic esophageal mucosa down to the level of the GE junction was found (Figure 1).

CT scan of the chest and upper abdomen demonstrated a well-circumfused mass at the lower thoracic esophagus with lipoma like density (-30.84 Hounsfield Units). No other lesions or enlarged lymph nodes at the lower mediastinum were found (Figure 2).

The patient was put on parenteral nutrition and nil by mouth and on the 7th post admission day a second upper GI endoscopy clearly demonstrated an esophageal submucosal polypoid mass originating from the wall of the lower esophagus 4-5 cm above the GE junction. The tumor was covered by normal esophageal mucosa with

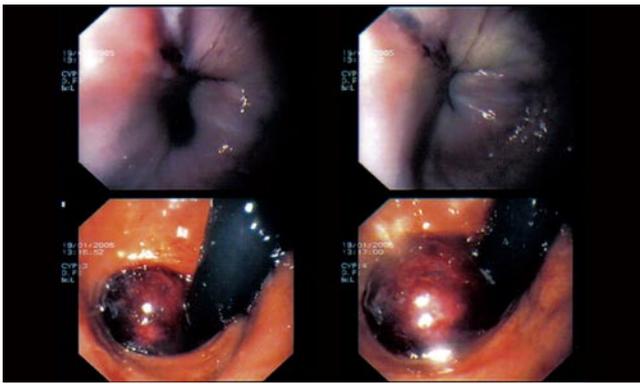


Figure 1 upper GI endoscopy A big hemorrhagic polypoid lesion as a haematoma in the hiatus hernia at upper GI endoscopy



Figure 2 Intraluminal mass with a lipoma-like density in the lower esophagus.

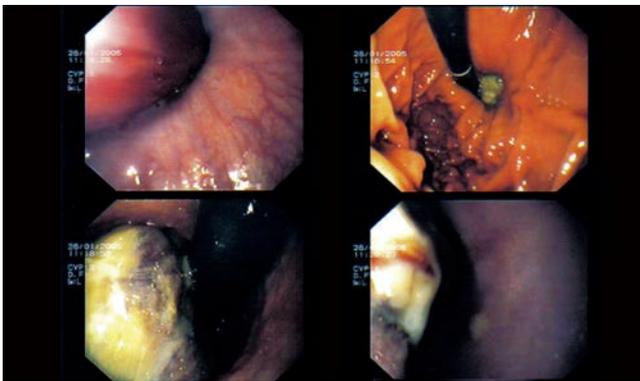


Figure 3 The lesion is covered by yellowish exudates at the second upper GI endoscopy.

central ulceration (Figure 3) and it seemed that it could be easily and intermittently intussuscepted through the GE junction into the stomach. A small sliding diaphragmatic hernia was also found. The stomach and the duodenum appeared to be normal. Multiple biopsies were taken especially from the ulcerated part of the tumor which failed to reveal any malignancy.

The patient was scheduled for an operation. Through a left posterior-lateral thoracotomy, a dilated lower thoracic esophagus with edematous muscle wall layers was found. The sliding diaphragmatic hernia was also noted. After a longitudinal incision just above the GE junction, the esophageal lumen was entered which was almost com-



Figure 4 Intraoperative view of the esophageal liposarcoma (A) and the specimen (B) (5 cm x 3.5 cm x 2 cm).

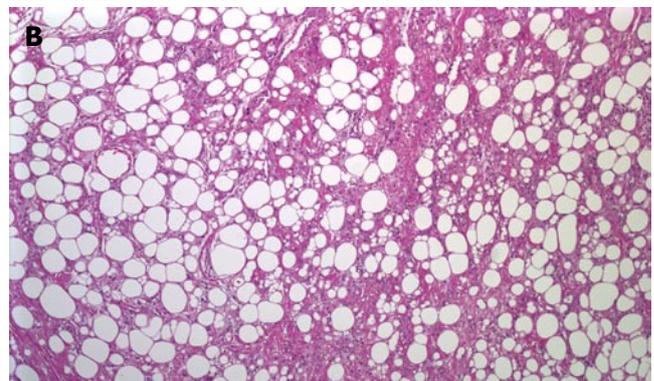
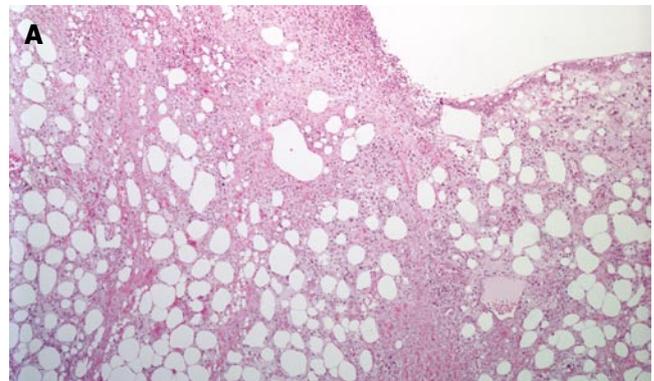


Figure 5 Neoplasm infiltrating smooth muscle layer of esophagus and ulcerating surface epithelium (A), HE 100x; and neoplasm consisting of differently-sized lipocytic element and multi-vacuolated lipoblasts (B), HE 400x. A small amount of sclerotic stroma was focally identified with the presence of atypical stromal cells.

pletely obstructed by a big polypoid submucosal mass. A central necrosis of the overlying esophageal mucosa

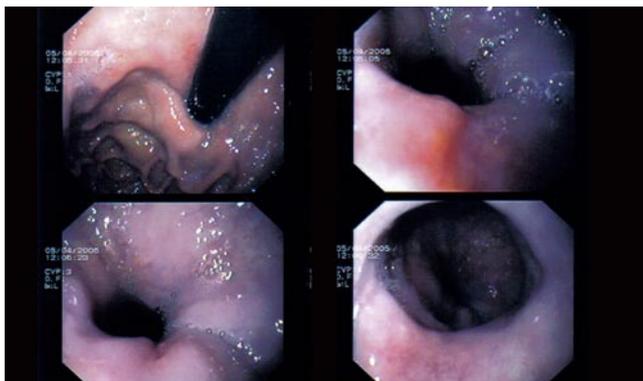


Figure 6 No evidence of tumor recurrence in gastrointestinal endoscopy after 6 mo of surgery.

revealed a lipoma like tumor which seemed to originate from the muscle layer of the esophageal wall, 5cm above the GE junction. We performed a local resection including a part of the esophageal muscle wall (Figures 4A and 4B). The esophageal incision was closed in 2 layers with interrupted absorbable sutures.

The postoperative period was uneventful, and the patient was discharged on the 8th postoperative day on a liquid - soft diet, with complete resolution of his preoperative symptoms.

Macroscopically the tumor was measured 5 cm × 3.5 cm × 2cm. Its surface was covered by normal esophageal mucosa measuring 3.5 cm × 1.5 cm with a central ulceration 1cm in diameter. The cut end of the mass revealed the adipocytic nature of the neoplasm and its color ranged from ivory to deep yellow with light brown areas. Histologically the neoplasm consisted predominantly of mature looking fat tissue which showed severe size and shape variation of adipocytic element with the presence of numerous multi vacuolated lipoblasts. It was divided by few focally sclerotic fibrous septa containing atypical stromal cells. Smooth muscle layer of the esophagus was extensively infiltrated close to the surgical margin (5mm). Surface epithelium was ulcerated. Areas of liponecrosis were often observed. The tumor had characteristic features of well-differentiated grade I liposarcoma with lipoma-like component (Figures 5A and 5B).

The patient was followed up and 6 mo after the operation, CT scan of the chest and upper abdomen and upper GI endoscopy showed no tumor recurrence or distal metastases (Figure 6).

DISCUSSION

Lipomatous tumors of the gastrointestinal tract are usually found in the distal ileum and the large bowel. Primary esophageal liposarcoma is exceedingly rare and slow growing tumor arising from mucosa and submucosa esophageal layers. The first case was described by Mansour *et al*^[2] in 1983. In the world literature 14 cases have been reported^[2-15]. The mean age varies between 43 and 73 years with a male: female ratio of 1.16:1. The clinical symptoms of esophageal lipomatous tumors include progressive

dysphagia, loss of weight, regurgitation, retrosternal pain, respiratory distress, odynophagia, sensation of a lump in the throat, hemorrhage, anemia, sudden death (asphyxia), vomiting (food or tumor fragments), fever and cough. The location of most tumors is the upper esophagus. There has been reported 5 myxoid, 1 pleiomorphic and 9 well-differentiated, with our case, esophageal liposarcomas^[3,5,7,8,15].

Barium swallowing, esophageal manometry, radiological and endoscopic examinations could be confused with usual esophageal pathologies, such as megaesophagus, achalasia or bezoars. The only diagnostic methods for these tumors are surgical excision and histological examination^[8,14,15].

Surgical treatment varies from simple enucleation or endoscopic resection to partial or total esophagectomy or trans-cervical, transthoracic, transgastric resections^[2-15]. Local recurrences can be decreased with post-operative radiation. The most reliable prognostic factors are liposarcoma grade, histological subtype, location and adequacy of surgical treatment^[8,10].

Local recurrence could take place in patients with well-differentiated liposarcomas who have a 5-year survival of 75 - 100%. Patients with other types of liposarcomas have a lower survival and a higher recurrence rates. The survival of the reported cases varies between 7 to 104 mo^[2,5,7,8,10].

Our case presented with acute pain of epigastrium, retrosternal sensation of burning, persistent nausea, vomiting and dysphagia. The differential diagnosis of several studies (barium swallow, esophagomanometry and esophagogram) confused us and included megaesophagus, achalasia, mediastinitis, haematoma or trauma from esophagomanometry. The measurement of tumor density with CT scan and secondary upper GI endoscopy showed that this mass was an esophageal lipomatous tumor. Although the biopsies were negative for malignancy we proceeded to tumour enucleation. Only surgical removal and histologic examination could diagnose esophageal malignancies such as liposarcoma. Our patient was followed up and 6 mo post-operation the CT scan of the chest and upper abdomen and upper GI endoscopy showed no tumor recurrence or distal metastases.

We believe that our case is considerable because of the rarity of esophageal liposarcoma. Esophageal lipoma or liposarcoma can be diagnosed if a patient has a history of slow growing esophageal mass with a low tumor density in CT in combination with surgical resection and histologic examination.

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Resolution of tuberculous biliary stricture after medical therapy

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Abstract

Tuberculosis (TB) is a very rare cause of biliary stricture that is difficult to diagnose and usually requires surgical intervention in order to rule out underlying malignant etiology. We report a 56-year-old man presented with jaundice, weight loss and poor appetite. Initial work up showed the dilated biliary system secondary to distal common bile duct stricture. Investigations to define the etiology of this stricture showed inconclusive brush cytology with absent abdominal masses and lymph nodes but enlarged mediastinal lymph nodes. Biopsy from these lymph nodes showed a non-caseating epithelioid granuloma with negative acid fast bacilli (AFB) stain. The patient had a dramatic response to empirical anti-tuberculosis therapy. Six weeks later, culture from lymph nodes was positive for *Mycobacterium tuberculosis*.

Three months later, follow-up cholangiogram showed complete resolution of the stricture with normalization of liver enzymes 6 mo after starting anti-tuberculosis therapy. Treatment was continued for 12 mo and the patient had a normal life with normal liver enzymes and regression of the mediastinal lymph nodes at the time when he was reported in this paper.

Although 16 cases of tuberculous biliary stricture are available in the English literature, up to our knowledge, this is the second published report of tuberculous biliary stricture, which resolved completely after medical therapy alone and the second reported case from the Middle East. This report emphasizes the importance of keeping TB as a possibility of biliary stricture in this part of the world.

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Key words: Biliary stricture; Tuberculosis; Hepatobiliary tuberculosis; Obstructive jaundice

Alsawat KE, Aljebreen AM. Resolution of tuberculous biliary stricture after medical therapy. *World J Gastroenterol* 2006;

INTRODUCTION

Although tuberculosis (TB) of the hepatobiliary system is not uncommon, it is an extremely rare cause of biliary stricture. Many cases of lymph node, gall bladder, duodenal and pancreatic tuberculosis are reported^[1-5], but only sixteen cases of biliary stricture caused by tuberculosis are available in the English literature^[6-16], and only one case has been reported from the Middle East^[13]. The main symptoms of TB biliary stricture including jaundice and weight loss are usually indistinguishable from those of other diseases such as cholangiocarcinoma. Although the presence of past history or chest X-ray changes of tuberculosis may raise the suspicion of this etiology, most of the reported cases are diagnosed based on surgical pathology. We report here a middle age gentleman who had tuberculous biliary stricture with an excellent outcome after anti-TB therapy alone.

CASE REPORT

A previously healthy 56-year-old Saudi male presented with a 4-mo history of fatigue, 15 kg weight loss, poor appetite, pruritus, progressive jaundice, dark urine, and pale stool. He had no history of fever, night sweat, alcohol or herbal medicine use. Past history was negative for TB, gall bladder or biliary disease or abdominal surgery.

Physical examination revealed that he was afebrile and thin with a body mass index of 19 kg/m², muscle wasting, deep yellow sclera, no palpable lymph nodes or stigmata of chronic liver disease. Abdominal examination showed palpable liver with a span of 9 cm and moderate ascites, and nor remarkable findings in other systems.

Liver enzymes showed a cholestatic pattern with 155 μmol/L total bilirubin, 102 μmol/L direct bilirubin, 352 u/L alkaline phosphatase, 77 u/L alanine aminotransferase and 27 g/L albumin. He had normal complete blood count with prolonged INR (1.7). Ascetic fluid analysis showed normal cell count, with high serum ascites albumin gradient of 18 g/L, suggestive of portal hypertension etiology. Gram, Ziehl-Neelsen (Z-N), routine and TB cultures from ascetic fluid as well as tuberculin skin test were

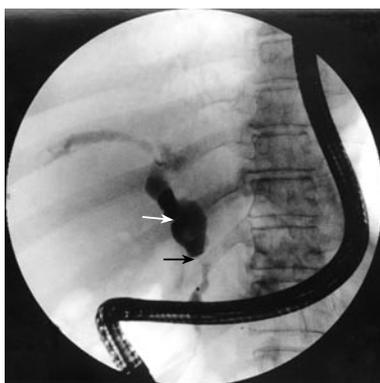


Figure 1 Initial ERCP showing dilated proximal biliary system (white arrow) with a tight short distal common bile duct stricture (black arrow).

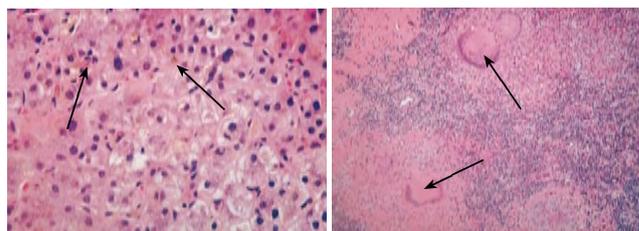


Figure 3 Hematoxylin and eosin (H and E) staining of liver tissue (A) and mediastinal lymph node (B) showing bile pigments (thick arrow) with bile duct proliferation (thin arrow) and inflammatory cell infiltrate and epithelioid granuloma with many langerhans cells but no caseation (arrows).

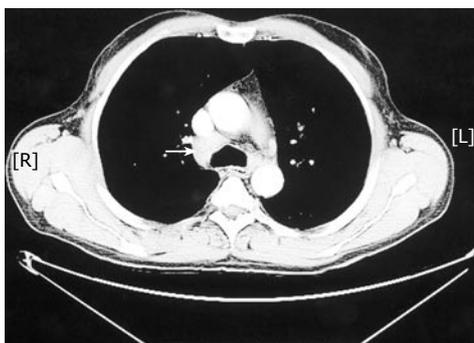


Figure 2 Computed tomography scan of the chest showing enlarged mediastinal (Para-tracheal, sub-carinal) lymph nodes (arrow).

Table 1 Liver function test on admission, two weeks on treatment and after stent removal showing complete normalization of biochemical changes

| | On admission | Two weeks on anti-TB treatment | After stent removal, three months on anti-TB treatment |
|----------------------------------|--------------|--------------------------------|--|
| Total bilirubin. (μmol/L) N:3-7 | 155 | 36 | 12 |
| Direct bilirubin. (μmol/L) N:0-5 | 102 | 24 | 5 |
| ALP (μ/L) N:50-136 | 359 | 95 | 154 |
| GGT (μ/L) N:15-85 | 200 | 43 | 53 |
| ALT (μ/L) N:20-65 | 77 | 21 | 22 |
| AST (μ/L) N:12-37 | 48 | 23 | 24 |
| Albumin(g/L) N:30-50 | 27 | 26 | 35 |

negative.

Chest X-ray was normal. Abdominal ultrasonography (US) showed features of cirrhosis with extra- and intra-hepatic biliary dilatation. The diameter of the common bile duct (CBD) was 12 mm. Doppler US revealed portal vein thrombosis but patent hepatic veins. The patient underwent endoscopic retrograde cholangiopancreatography (ERCP) which revealed no duodenal or ampullary mass, but single short tight irregular distal CBD stricture with dilated biliary tree (Figure 1). A plastic biliary stent was placed for drainage. Brush cytology was negative for malignant cells. Computed tomography (CT) scanning of the abdomen showed shrunken liver with no evidence of abdominal masses or lymphadenopathy, while CT chest showed multiple significant mediastinal lymph nodes (Figure 2).

Cholangiocarcinoma was found with probable mediastinal lymph node metastasis. However, given this location of lymph nodes, other possibilities such as lymphoma, tuberculosis or infiltrative diseases should be excluded.

Meanwhile a full workup for liver cirrhosis etiology showed only positive hepatitis B virus core antibodies (HBc-IgG). The other viral markers, anti-nuclear antibodies, anti-smooth muscles antibodies and anti-mitochondrial antibodies were negative. Alpha-1-antitrypsin, serum copper, ceruloplasmin and iron profile were normal. Liver biopsy showed a minimal bile duct proliferation with portal tract fibrosis and cholestasis but no granuloma or malignant cells (Figure 3A). Upper gastrointestinal endoscopy showed grade-4 esophageal varices with congestive gastropathy.

These results indicated that the most likely cause of

ALP, Alkaline phosphatase; GGT, Gamma-glutamyltransferase. ALT, Alaninaminotransferase; AST, Aspartate aminotransferase. N, Normal laboratory values

liver cirrhosis was previous exposure to hepatitis B infection.

Then patient underwent mediastinoscopy with lymph node excisional biopsy, which showed epithelioid granuloma with numerous langerhans cells but no caseation (Figure 3B). Z-N stain was negative for acid fast bacilli and biopsy was sent for TB culture.

Based on the result of lymph node biopsy, we decided to start empirical first line anti-tuberculosis (anti-TB) drugs in a full dose (isoniazid, 300 mg/d; rifampicin, 600 mg/d; ethambutol, 1 g/d; pyrazinamide, 1.5 g/d) while waiting TB culture result and observing the response. The patient tolerated well anti-TB therapy and had dramatic clinical and biochemical improvements (Table 1). Six weeks later, lymph node biopsy culture showed *Mycobacterium tuberculosis* which was sensitive to all these medications.

Three months after starting the anti-tuberculous therapy, repeated ERCP showed complete resolution of the biliary stricture with normal extra- and intra-hepatic biliary tree, and the biliary stent was removed (Figure 4).

During the follow-up, significant improvement was found in the ascites. The patient required small doses of diuretics to control it, and had prophylactic variceal band ligation. Additional Doppler ultrasound showed recanalization of the portal vein. The patient had a normal life and gained weight (around 25 Kg) with normal liver



Figure 4 ERCP showing complete resolution of the previous distal common bile duct stricture three months after anti-tuberculous therapy.

enzymes and function at the time when he was reported in this paper.

DISCUSSION

Tuberculous biliary stricture as a cause of obstructive jaundice is extremely uncommon. It involves all parts of the biliary tree, namely the intrahepatic biliary radicles, right and left hepatic ducts, common hepatic duct, cystic duct and CBD^[6-16].

Obstructive jaundice due to hepatobiliary TB is most often attributed to mechanical obstruction of the biliary tract by tuberculous lymph nodes or mass lesions. Our patient had no tuberculous lymph nodes or mass lesions, but the stricture was due to a secondary endoluminal inflammatory process.

The clinical and cholangiographic features of tuberculous biliary stricture are usually not helpful in differentiating tuberculosis from other common causes of endoluminal biliary stricture such as primary sclerosing cholangitis or cholangiocarcinoma. Alvarez and Sollano^[17] have proposed the characteristic cholangiographic patterns of hepatobiliary tuberculosis, including a tight hilar stricture with dilated intrahepatic ducts, a long smooth stricture involving the distal bile duct, pruning of the distal intrahepatic ducts, and sclerosing cholangitis-like changes. However, the cholangiographic findings in our patient could not be differentiated from those caused by other factors.

Most of the reported cases of tuberculous biliary stricture are diagnosed until laparotomy is performed. The histopathologic findings of tuberculosis include caseating granulomatous inflammation and Langhans giant cells^[8,14]. However, in some cases the diagnosis is achieved by detection of acid fast bacilli (AFB) after staining or culture in the biliary fluid aspirate during ERCP^[7], but the yield of these tests is low. PCR technique for *Mycobacterium tuberculosis* from biliary fluid may be helpful^[13,16]. Prasad and Pandey^[15] reported that AFB of the aspirated bile can diagnose tuberculous biliary stricture.

In our patient, both diagnosis and management were challenging. The diagnosis was difficult because of clinical presentations mimicking other common etiology, lack of constitutional symptoms of tuberculosis such as fever and night sweat, absence of past history of tuberculosis, negative tuberculin skin test, lack of radiological evidence of old or active tuberculosis. However, the presence of mediastinal lymphadenopathy, an unusual site for a meta-

static cholangiocarcinoma, raises the possibility of other diseases such as tuberculosis, lymphoma or sarcoidosis. Therefore, mediastinoscopy with excisional biopsy from the mediastinal lymph nodes is essential for confirming the diagnosis of tuberculosis as the likely cause of biliary stricture and excluding other possibilities.

The challenge in the management is the high risk of anti-TB hepatotoxicity, especially in the setting of liver cirrhosis and the uncertainty of the diagnosis. The possibility of cholangiocarcinoma or lymphoma can be completely ruled out based on the dramatic clinical, biochemical and radiological responses to Anti-TB drugs as well as the positive TB culture.

In most of the reported cases, the biliary stricture does not resolve with medical therapy alone and requires surgical intervention and biliary metallic stent placement^[7,13]. However, to our knowledge, biliary stricture is completely resolved only in one case after medical therapy without surgery or permanent biliary drainage procedure^[16]. In our patient, the biliary stricture was completely resolved within 3 mo of anti-TB therapy without any further intervention. The reason for this is likely due to no permanent fibrotic changes.

In conclusion, tuberculous biliary stricture can be completely resolved after medical therapy alone, highlighting that the diagnosis of such cases is often difficult and a high index of suspicion must be kept in mind especially in areas where TB is relatively common.

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A case of huge primary liposarcoma in the liver

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Abstract

Primary liver liposarcoma is a rare disease. Because of its rarity, the knowledge of the clinical course, management, and prognosis of primary liver liposarcoma are all limited for clinicians. A 61-year-old female patient who suffered from a huge primary liposarcoma in the central portion of the liver had the clinical presentations of fever, nausea, vomiting, jaundice, and body weight loss. The huge tumor was resected successfully. However, the tumor recurred repeatedly and she had repeated hepatectomies to remove the tumor. The tumor became aggravating after repeated surgeries. Eventually, the patient had cervical spinal metastasis of liposarcoma and she survived for 26 months after liver liposarcoma was diagnosed. Although the tumor may become aggravating after repeated surgeries, repeated hepatectomies are still the best policy to achieve a long-term survival for the patients.

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Key words: Liver liposarcoma; Hepatectomy

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INTRODUCTION

Liver neoplasm is not an uncommon disease in Asian countries. Cavemous hemangioma, adenoma, and focal nodular hyperplasia are the most common benign tumors in the liver. Hepatocellular carcinoma and cholangiocarcinoma are the most common malignant ones. Liver tumors derived or transformed from other mesenchymal cells, however, are rare. Therefore, the

precise diagnosis of a benign tumor other than cavernous hemangioma, adenoma and focal nodular hyperplasia, or a malignant tumor other than hepatocellular carcinoma and cholangiocarcinoma is difficult.

Liposarcoma is not an uncommon malignant mesenchymal neoplasm. However, it is extremely rare in the liver. To our knowledge, most liposarcomas are found in lower extremities, shoulders and retroperitoneum^[1]. Only few cases of primary liver liposarcoma have been reported^[2]. Furthermore, most of the articles describing liver liposarcoma focus only on histopathologic and image studies. The clinical course and prognosis of primary liver sarcoma are rarely mentioned. Hence, we have presented a case of a primary huge liver liposarcoma with the clinical course and have described the outcome of the patient.

CASE REPORT

A 61-year-old woman was admitted because she had fever, nausea, vomiting, jaundice, and tea-colored urine for a week. She had lost 5 kg of body weight within a month. Physical examination revealed that she had hepatomegaly, icteric sclera, and a palpable mass over the right upper quadrant of the abdomen. Laboratory studies showed 61 mg/L total bilirubin, 702 u/L alkaline phosphate, 277 u/L gamma-glutamyl-transpeptidase, 61 u/L aspartate aminotransferase, and 18 u/L alanine aminotransferase. Virologic examination revealed that she was not infected with hepatitis B or C virus. Serum levels of α -fetoprotein and CEA were measured and both were within the normal limits. However, CA-199 was up to 92.2 U/mL (normal: <37 U/mL at this hospital). Abdominal ultrasonography showed a huge lobulated cystic lesion in the central portion of the liver. Computed tomography (CT) scan was done and demonstrated that a huge cystic lesion was located at segments 4, 5, and 8, and bilateral intrahepatic ducts were compressed (Figure 1). Aspiration cytology was done but failed to demonstrate any malignant cells. Since this patient had fever and her bilateral intrahepatic ducts were compressed by the tumors, biliary tract infection could not be ruled out as she was treated with antibiotics first. Although the tumor was difficult to locate, surgical resection was still feasible according to the imaging studies. Therefore, surgical resection of the tumor was done and a three-segmentectomy with the preservation of bilateral main intrahepatic ducts was performed. Grossly, the tumor was a cystic tumor, 11 cm \times 11 cm \times 13 cm in size, with multiple septa (Figure 2). Microscopically, the tumor showed the picture of a myxoid liposarcoma (Figure 3). Postoperative course was uneventful and she was dis-



Figure 1 A low-attenuation cystic mass located in segments 4, 5, and 8 with bilateral biliary tract compression in a non-cirrhotic liver by CT.

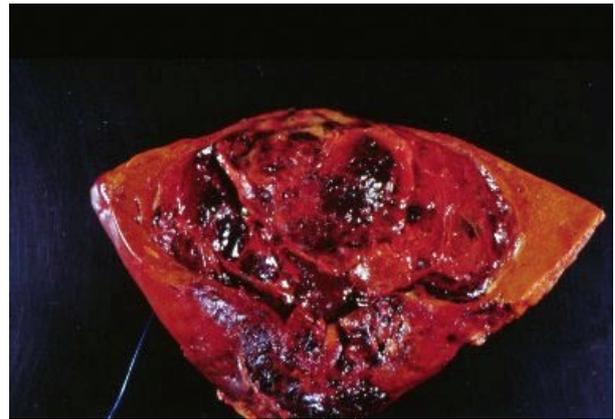


Figure 2 A lobulated liver liposarcoma with intra-tumor hemorrhage.

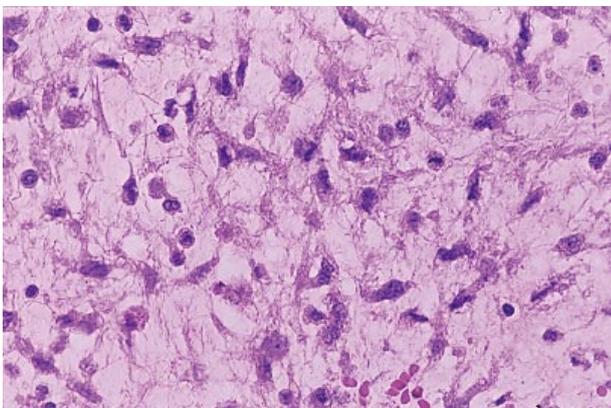


Figure 3 Numerous lipoblasts and capillaries in the myxoid liposarcoma (hematoxylin-eosin staining, 400 ×).

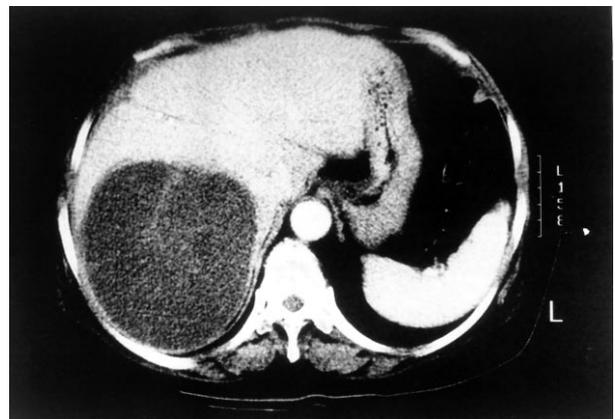


Figure 4 A big recurrent liver liposarcoma locating in the right lobe of the liver by CT.

charged on the tenth postoperative day. She was followed up at our out patient clinics and liver ultrasonography was performed every 2 or 3 mo.

A big tumor, 9.5 cm × 10 cm × 12.4 cm in size, recurred in the right lobe of the liver 10 mo after the first surgery although liver sonography was performed regularly and no tumor was found in previous examinations (Figure 4). Hepatectomy was performed again to excise the tumor. Pathologic pictures showed that the tumor was a recurrent myxoid liposarcoma. The patient was then discharged and was followed up at our outpatient clinics. Nevertheless, a tumor, 7 cm × 9 cm × 12 cm in size, occurred again in the liver 11 months after the second surgery. Whole body bone scan with Tc-99m MDP was performed and showed negative for bony metastasis. Thereafter, hepatectomy was performed again to excise the tumor. The pathologic pictures showed undifferentiated sarcoma of the liver (Figure 5). However, the patient complained about neck pain 2 wk after the third hepatectomy. A cervical spine metastasis (C3-C4) was noted. Laminectomy was performed to remove the spinal tumor and release the spinal cord compression.

Although aggressive surgical treatments were employed to treat this liposarcoma, tumor recurred in the liver 2 mo

after the last surgery. Since the patient could not tolerate further surgical interventions, radiotherapy was performed. But the tumor did not respond to radiotherapy, and her condition deteriorated. The patient died of liver failure and aspiration pneumonia five months after the last hepatic resection surgery.

DISCUSSION

Primary liver liposarcoma is rare. The experience for a surgeon or a gastroenterologist is also limited. In this case study, the patient survived for 26 mo after an aggressive treatment. Wolloch *et al*^[3] described the first liver myxoid liposarcoma patient. The patient underwent a right hemihepatectomy and survived for 46 d only after the surgery. Subsequently, only few cases have been described^[2]. Moreover, the clinical course and management of primary liver liposarcoma are rarely mentioned in these reports (Table 1). Therefore, the knowledge of primary liver liposarcoma is still limited. The results of surgical resections are unknown. The long-term prognosis for liver liposarcoma is unknown either.

Clinical course and management of primary liver liposarcoma are scarcely mentioned in previous reports because the patients were too old or had serious underlying

Table 1 Location, management, and result of previously reported cases of primary liver liposarcoma in adults

| Case source | Age (yrs) | Sex | Location | Management | Results |
|-------------------------------------|-----------|-----|----------------------|------------------------------------|-----------------------------------|
| Wolloch <i>et al</i> ^[3] | 22 | F | Right lobe | Right lobe hepatectomy | Survival 46 d |
| Kim and Reyes ^[6] | 86 | M | Capsule | Supportive treatment | NM |
| Kim <i>et al</i> ^[7] | 30 | F | Right lobe | Right lobe hepatectomy | Tumor free for 10 mo |
| Aribal <i>et al</i> ^[8] | 48 | F | Hilum | Chemotherapy | NM |
| Nelson <i>et al</i> ^[2] | 54 | F | Left lobe right lobe | Exploratory laparotomy with biopsy | Post-operative bleeding and death |
| Current study | 61 | F | Right lobe | Right lobe hepatectomy | Survival 27 mo |

NM: not mentioned.

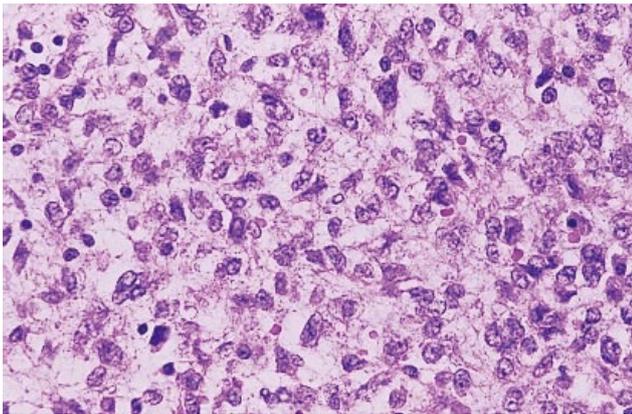


Figure 5 A high grade of undifferentiated liposarcoma composed of spindle cells with marked nuclear pleomorphism (hematoxylin-eosin staining, $\times 400$).

ing diseases to receive a surgery, or the tumor was big-sized or located in a position difficult for complete resection. In this case, curative hepatectomy was performed although the tumor was huge. According to the clinical course, liver liposarcoma has a high rate of recurrence, which is similar to those at other sites^[1,4]. Aggressive surgery can prolong the life of patients. Thus, this case provides a good experience about the clinical course of primary liver liposarcoma.

Early diagnosis of liver liposarcoma is not easy. Liver is the largest organ in the abdomen, and symptoms and signs of a tumor in the liver are usually trivial unless the tumors are large. The usual symptoms and signs of primary liver liposarcoma include jaundice, fever, nausea, vomiting, abdominal fullness, right upper quadrant pain, and body weight loss^[2]. Most of the symptoms are caused either by displacement or compression of nerves, vessels, biliary tract, and intestinal structure. Our case had similar clinical presentations because of the massive effect of tumor and central location of the tumor to compress biliary tracts. Abdominal ultrasonography usually is a good tool to do preoperative screening and postoperative follow-up. In this case, abdominal ultrasonography was performed

regularly at an interval of 12 wk. However, the recurrent tumor was always large when the tumor was found by ultrasonography. This implies that recurrent tumor grows rapidly between two ultrasonography examinations. Computed tomography is still the best tool to evaluate the resectability of liver liposarcoma before surgery.

Five major histologic categories of liposarcoma are mentioned^[5] and patients with myxoid or well-differentiated type have a lower local recurrence rate than other types^[1]. In our case, surgical specimens showed myxoid liposarcoma in the first and second time. However, the tumor exhibited high grade of undifferentiated sarcoma in the third time. The interval to recurrence in the first and second time was 10 mo and 11 mo, respectively, but recurrence interval in the third time reduced to 2 mo. The tumor pathology became aggravating after repeated surgery.

In conclusion, primary liposarcoma of the liver is rare. The experience in dealing with this malignant neoplasm is limited. In our experience, the tumor becomes aggravating with the time course or after repeated surgeries, and resists to radiotherapy. Curative and aggressive hepatectomies are still the best policy to achieve a long-term survival of the patients.

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Gastroenterology Association
May 20-25, 2006
Loas Angeles Convernition Center, California
www.ddw.org

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www.ddw.org

Annual Postgraduate Course
May 25-26, 2006
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- 6 **Morse SS**. Factors in the emergence of infectious diseases. *Emerg Infect Dis* serial online, 1995-01-03, cited 1996-06-05; 1(1):24 screens. Available from: URL: <http://www.cdc.gov/ncidod/EID/eid.htm>

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Statistical expression

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| Number | Nonstandard | Standard | Notice |
|--------|--|--|--|
| 1 | 4 days | 4 d | In figures, tables and numerical narration |
| 2 | 4 days | four days | In text narration |
| 3 | day | d | After Arabic numerals |
| 4 | Four d | Four days | At the beginning of a sentence |
| 5 | 2 hours | 2 h | After Arabic numerals |
| 6 | 2 hs | 2 h | After Arabic numerals |
| 7 | hr, hrs, | h | After Arabic numerals |
| 8 | 10 seconds | 10 s | After Arabic numerals |
| 9 | 10 year | 10 years | In text narration |
| 10 | Ten yr | Ten years | At the beginning of a sentence |
| 11 | 0,1,2 years | 0,1,2 yr | In figures and tables |
| 12 | 0,1,2 year | 0,1,2 yr | In figures and tables |
| 13 | 4 weeks | 4 wk | |
| 14 | Four wk | Four weeks | At the beginning of a sentence |
| 15 | 2 months | 2 mo | In figures and tables |
| 16 | Two mo | Two months | At the beginning of a sentence |
| 17 | 10 minutes | 10 min | |
| 18 | Ten min | Ten minutes | At the beginning of a sentence |
| 19 | 50% (V/V) | 500 mL/L | |
| 20 | 50% (m/V) | 500 g/L | |
| 21 | 1 M | 1 mol/L | |
| 22 | 10 μM | 10 μmol/L | |
| 23 | 1N HCl | 1 mol/L HCl | |
| 24 | 1N H ₂ SO ₄ | 0.5 mol/L H ₂ SO ₄ | |
| 25 | 4rd edition | 4 th edition | |
| 26 | 15 year experience | 15- year experience | |
| 27 | 18.5 kDa | 18.5 ku, 18 500u or M:18 500 | |
| 28 | 25 g.kg ⁻¹ /d ⁻¹ | 25 g/(kg·d) or 25 g/kg per day | |
| 29 | 6900 | 6 900 | |
| 30 | 1000 rpm | 1 000 r/min | |
| 31 | sec | s | After Arabic numerals |
| 32 | 1 pg L ⁻¹ | 1 pg/L | |
| 33 | 10 kilograms | 10 kg | |
| 34 | 13 000 rpm | 13 000 g | High speed; g should be in italic and suitable conversion. |
| 35 | 1000 g | 1 000 r/min | Low speed. g cannot be used. |
| 36 | Gene bank | GenBank | International classified genetic materials collection bank |
| 37 | Ten L | Ten liters | At the beginning of a sentence |
| 38 | Ten mL | Ten milliliters | At the beginning of a sentence |
| 39 | umol | μmol | |
| 40 | 30 sec | 30 s | |
| 41 | 1 g/dl | 10 g/L | 10-fold conversion |
| 42 | OD ₂₆₀ | A ₂₆₀ | "OD" has been abandoned. |
| 43 | One g/L | One microgram per liter | At the beginning of a sentence |
| 44 | A260 nm ^b P<0.05 | A ₂₆₀ nm ^a P<0.05 | A should be in italic. In Table, no note is needed if there is no significance instatistics: ^a P<0.05, ^b P<0.01 (no note if P>0.05). If ther is a second set of P value in the same table, ^c P<0.05 and ^d P<0.01 are used for a third set: ^a P<0.05, ⁱ P<0.01. |
| 45 | *F=9.87, [§] F=25.9, [#] F=67.4 | ¹ F=9.87, ² F=25.9, ³ F=67.4 | Notices in or under a table |
| 46 | KM | km | kilometer |
| 47 | CM | cm | centimeter |
| 48 | MM | mm | millimeter |
| 49 | Kg, KG | kg | kilogram |
| 50 | Gm, gr | g | gram |
| 51 | nt | N | newton |
| 52 | l | L | liter |
| 53 | db | dB | decibel |
| 54 | rpm | r/min | rotation per minute |
| 55 | bq | Bq | becquerel, a unit symbol |
| 56 | amp | A | ampere |
| 57 | coul | C | coulomb |
| 58 | HZ | Hz | |
| 59 | w | W | watt |
| 60 | KPa | kPa | kilo-pascal |
| 61 | p | Pa | pascal |
| 62 | ev | EV | volt (electronic unit) |
| 63 | Jonle | J | joule |
| 64 | J/mmol | kJ/mol | kilojoule per mole |
| 65 | 10×10×10cm ³ | 10 cm×10 cm×10 cm | |
| 66 | N·km | KN·m | moment |
| 67 | x±s | mean±SD | In figures, tables or text narration |
| 68 | Mean±SEM | mean±SE | In figures, tables or text narration |
| 69 | im | im | intramuscular injection |
| 70 | iv | iv | intravenous injection |
| 71 | Wang et al | Wang <i>et al.</i> | |
| 72 | EcoRI | EcoRI | <i>Eco</i> in italic and RI in positive. Restriction endonuclease has its prescript form of writing. |
| 73 | Ecoli | <i>E.coli</i> | Bacteria and other biologic terms have their specific expression. |
| 74 | Hp | <i>H pylori</i> | |
| 75 | Iga | Iga | writing form of genes |
| 76 | igA | IgA | writing form of proteins |
| 77 | ~70 kDa | ~70 ku | |

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Etiology and consequences of thrombosis in abdominal vessels

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Abstract

The thrombophilia which can be either congenital or acquired in adult life has major implications in the abdominal vessels. The resulting portal vein thrombosis, Budd-Chiari syndrome and mesenteric vein thrombosis have a variety of consequences ranging from acute abdomen to chronic hepatomegaly and even totally asymptomatic patient in whom the only finding is pancytopenia. The complications like esophageal varices, portal gastropathy, ascites, severe hypersplenism, liver failure requiring liver transplantation are well known. Interesting features of collateral venous circulation showing itself as pseudocholeangioma sign and its possible clinical reflection as cholestasis are also known from a long time. The management strategies for these complications of intraabdominal vessel thrombosis are not different from their counterpart which is cirrhotic portal hypertension, but the prognosis is unquestionably better in former cases. In this review we presented and discussed the abdominal venous thrombosis, etiology and the resulting clinical pictures. There are controversial issues both in nomenclature, and management including anticoagulation problems and follow up strategies. In light of the current knowledge, we discussed some controversial issues in literature and presented our experience and our proposals about this group of patients.

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Key words: Portal vein thrombosis; Pseudocholeangioma sign; Thrombophilia; Budd-Chiari syndrome; Mesenteric vein thrombosis

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INTRODUCTION

The thrombosis in the major vessels of abdomen causes a wide spectrum of clinical pictures ranging from a totally asymptomatic patient to a patient with acute abdominal pain and even impending liver failure in patients with underlying chronic liver disease. As liver is the main organ of synthesis of many essential proteins in the body, it bears the burden of thrombosis of major vessels in the abdomen whether this may be a partial or total thrombosis with resulting liver disease, portal hypertension and cirrhosis requiring different management strategies including liver transplantation.

In this clinical setting, the thrombosis of the abdominal vessels has a special place in medicine since the clinicopathological pictures and courses of these processes are usually heterogenous, protean and fluctuant in nature. In general practice physicians pay extra attention to the thrombosis of coronary, pulmonary, mesenteric or cerebral circulation but not to abdominal venous circulation. We believe that the thrombotic occlusion of all major vessels of abdominal cavity has severe clinical consequences and chronic complications.

In this review we will describe thrombophilic conditions and discuss the potential consequences of thrombosis in the major abdominal vessels with potential clinical implications.

THROMBOPHILIA

Normal coagulation hemostasis involves the interaction of an initial vascular reaction (vasoconstriction), thrombocytic activation (white clot formation) and formation of thrombin via activation of coagulation cascade. The balance between the forces favoring formation of a clot and forces against it is the normal state which is controlled with very delicate systems. Although very complicated and several mechanisms play a role in the vascular and thrombocytic steps of clot formation, these steps are beyond the scope of this review and will not be discussed further.

Thrombophilia is a term which is proposed as an opposite term against hemophilia. Thrombophilia can be defined as a disturbed state of the coagulation-fibrinolysis balance in favor of thrombosis formation (congenital or acquired in adult life) in which thrombosis (in arterial and/

or venous vasculature) is observed more frequently than normal population. For the gastroenterologists and surgeons, the congenital and acquired causes of the thrombophilia are important not only due to their potential risks to patients' lives but also their preventability with the advent of genetic tests and surveillance and their treatability with new invasive techniques and new anticoagulant drugs.

Thrombophilia can be grouped under two major headlines: inherited and acquired.

CAUSES OF INHERITED THROMBOPHILIA

Factor V Leiden mutation (FVLM)

The most common cause of inherited thrombophilia is FVLM. This mutation was firstly defined by Bertina *et al*^[1] in 1994, after studies of Dahlback and colleagues^[2] who showed a resistance factor against activated protein C (APC). The prevalence of this mutation differs between populations throughout the world. The prevalence of this mutation is very heterogenous that some native populations of Africa, America and Asia show no mutation whereas in some districts of Scandinavian peninsula the prevalence rises to 15%. In Turkey, the prevalence is about 8-9% and this prevalence rate decreases going west from Asia Minor towards Europe to 2-4%^[3].

Factor V (FV) is synthesized in liver and megakaryocytes which must be activated into form of FVA to play a crucial role in prothrombinase complex along with activated factor X to produce thrombin. The mutation involves a point at the 1691st nucleotide of the exon 10 in first chromosome making G-C change. This results in a change in aminoacid sequences which is depicted as A506G (Arginine at 506 changed by Glutamine). The mutation containing FVA is resistant to degradation by protein C (which is a natural anticoagulant protein) and undegraded factor rises over time increasing the risk of uncontrolled thrombin and therefore thrombus formation. The diagnosis of FVLM depends partially on the activated protein C resistance (APCR) as a screening tool but direct PCR test to detect mutation can also be applied. APCR is a standard test which is widely available in most laboratories but modified APCR has a very high level of sensitivity and specificity and should be applied to patients who are on warfarin treatment, anti-phospholipid syndrome patients, pregnant women, protein S (PS) deficiency and patients with high levels of factor VIII^[4].

When compared with normal population, the impact of FVLM on thrombosis formation is a risk about 3-7 times higher in heterozygotes and 50-100 times higher in homozygotes.

Prothrombin mutation (PM)

PM is firstly defined in 1996 by Poort *et al*^[5]. The mutation involves a base pair change in the 20210th position (guanosine vs. adenosin) resulting in excessive generation of prothrombin which forms excessive procoagulant accumulation^[6]. This mutation has a general population prevalence of about 2% compared to the prevalence increased to 6% in patients who had their first attack of venous thrombosis. Like FVLM, the genetic basis is also important for the prevalence. In African and

Asian populations the PM is rare when compared to Caucasians^[7].

Protein C (PC) and protein S (PS) deficiency

PC is synthesized in liver by a vitamin K dependent mechanism. During coagulation cascade, this protein is activated by thrombin into its active form, namely activated PC (APC). PC is encoded in chromosome 2 and up to date about 160 mutations have been defined^[4,8-10]. The PC activity is usually determined by functional tests. The heterozygote PC deficiency reveals itself by PC activity less than 50%, whereas in homozygote deficiency PC activity is below 5%. The prevalence of PC deficiency is about 0.2% in general population and in the population who had first venous thrombosis attack the prevalence is found to be about 3%^[11]. An important factor to point out is that the acquired PC deficiency occurs in patients with liver disease (due to decreased synthetic capacity), oral anticoagulation treatment and acute thrombosis cases (due to utilization).

PS is naturally a cofactor of activated PC during inactivation processes of activated factors V and VIII. This protein is synthesized from liver, endothelial cells, megakaryocytes and in testis by vitamin K dependent reactions. There are many mutations responsible for PS deficiency so that genotyping and analysis for a suspected PS deficiency is not practical. The screening for PS deficiency is functional activity testing. The protein S deficiency is accepted as a weak risk factor for thrombosis formation which is about 2 times more than normal population. The importance of liver disease in assessing these natural anti-coagulant deficiencies is discussed later.

Antithrombin (AT) deficiency

AT is a natural serine protease inhibitor with a very potential controlling functions over coagulation cascade. AT inhibits the steps of coagulation depending on mainly thrombin and less importantly activated factors IX, X, XI, and XII. In the presence of heparin or heparin like molecules the inhibitor function is 1000 times more potent^[12]. The prevalence of deficiency of AT in the general population is about 0.02-0.2%, while it accounts for 1% of venous thrombosis etiologies^[11,13].

CAUSES OF ACQUIRED THROMBOPHILIA

In young adults, the annual risk of venous thrombosis is 1/10000 for event/person but this figure increases to 1/100 for event/person in patients over 70 years of age^[14]. Age is considered as an independent risk factor for venous thrombosis mainly due to inactivity (related with venous congestion), comorbid illnesses related with age and degenerative changes in the vasculature that occur with aging^[15].

Malignant disease is also an important cause of thrombosis. Cancer patients have a crude risk of 10-20% to have venous thrombosis in the rest of their lives. The thrombosis related deaths row in the second place in all causes of deaths in this patient population^[16-18]. Myeloproliferative disorders (MPD) merit a special discussion in this section. MPD forms a group of special blood disorders which may be termed as half-malignant due to their natural course

Table 1 Relative risks and comparison of common thrombophilic conditions^[21,22].

| Thrombophilic condition | Relative risk of thrombosis |
|--|-----------------------------|
| Inherited | |
| Heterozygote deficiency of natural anticoagulants (PC, PS, AT) | 10-fold |
| Heterozygote FVLM | 5-8 fold |
| Homozygote FVLM | 50-80 fold |
| Heterozygote PM | 2-4 fold |
| Homozygote PM | 10-fold |
| Acquired | |
| Oral contraceptive use | 4-fold |
| Surgery | 6-fold |
| Immobilization | 11-fold |
| Anti-phospholipid syndrome | 10-fold |
| Combined | |
| Oral contraceptive use + Heterozygote FVLM | 35-fold |

and this group of patients frequently suffer from venous thrombosis. Thrombocytosis and the increased hematocrit which are natural characteristics of this disorders which also cause thrombosis in the venous systems^[19].

Other well known acquired thrombophilic conditions are surgery (orthopedic and neurosurgery), drugs (i.e., oral contraceptives, hormone replacement treatment, tamoxifen, L-Asparaginase), antiphospholipid syndrome, pregnancy and Behcet's disease^[20].

The relative risks of common thrombophilic conditions^[21,22] are shown in Table 1.

PORTAL VENOUS THROMBOSIS: ETIOLOGY, CLINICAL CONSEQUENCES

Portal vein thrombosis (PVT) refers to the total or near-total obstruction of blood flow secondary to thrombus formation. This thrombus may extend towards liver involving intrahepatic portal veins or may extend distally to involve splenic veins or mesenteric veins. On some occasions extensive involvement of all of these vessels may occur with an increased risk of intestinal ischemia. Therefore, the involved segment of portal venous system and the degree of compensatory mechanisms determine the clinical presentation. Although PVT has been observed most commonly in the setting of cirrhosis, discussion presented in the text from this point specifies chronic, non-cirrhotic PVT unless otherwise specified.

PVT has considerable consequences for the liver. Upon cessation of blood flow liver loses about two thirds of its blood supply. Interestingly, while the acute arterial blockage usually results in severe hepatic failure or death, this condition is tolerated well and the patients are almost asymptomatic due to compensatory mechanisms. First compensatory mechanism is the well known "arterial vasodilation" of the hepatic artery (which is a vascular reflex seen in every dual-vessel supplied organ) also observed during portal vein clamping in liver surgery^[23]. This "arterial rescue" stabilizes the liver functions at a normal level in the very acute stages of PVT. The second rescue mechanism is the "venous rescue" involving rapid

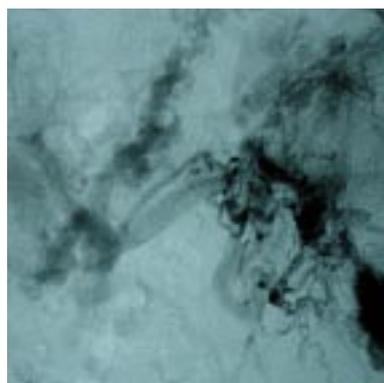


Figure 1 Conventional splenoportography showing a case of portal cavernomatous transformation with portosystemic collaterals, and extensive esophageal-gastric varicose veins.

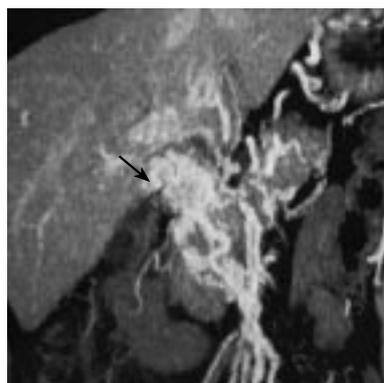


Figure 2 CT-angiography of portal system. Arrow shows the portal cavernomatous transformation with portosystemic collaterals.

development of collateral vessels to bypass the obstructed segment. These vessels begin to form in a few days after the obstruction and organize into a cavernomatous transformation (Figures 1 and 2) (see later section for detailed discussion) in 3-5 wk^[24,25]. We believe that when symptomatic, acute PVT rather be called "acute mesenteric thrombosis" due to extensive co-involvement of the superior mesenteric vein and branches. This condition has very acutely developing deleterious effects over intestinal circulation compromising the patient's life before development of portal hypertension and its consequences.

Besides these compensatory mechanisms, liver bears the burden of decreased blood to some extent. The decreased portal blood flow stimulates apoptosis in hepatocytes of rats when portal vein is obliterated in a gradual fashion^[26] and increases mitotic activity of hepatocytes in the unaffected lobe. The latter effect is well known from selective pre-surgery portal vein obliteration performed in an intention to stimulate the hypertrophy of the opposite lobe of the liver used in cancer surgery. Gradual loss of hepatic mass may be responsible for the occurrence of mild to moderate degree of hepatic synthetic dysfunction observed in advanced stages.

Etiology

PVT has various causes. The design of the studies and the parameters evaluating the cause of PVT have changed in parallel to the evolution in medical and genetic technology. In previous studies between 1979-1997, PVT were mostly attributed to trauma (5-17%), intra-abdominal sepsis (5-36%), umbilical sepsis (5-12%)^[27] (important cause of PVT in children), pancreatitis (4-5%) and prothrombotic

Table 2 Role of genetic thrombophilic factors in development of PVT

| Thrombophilic factor | Chamourad <i>et al</i> ^[41] | Egesel <i>et al</i> ^[40] | Janssen <i>et al</i> ^[39] | Denninger <i>et al</i> ^[33] | Primignani <i>et al</i> ^[38] |
|----------------------|--|-------------------------------------|--------------------------------------|--|---|
| <i>n</i> | 10 | 32 | 92 | 36 | 65 |
| FVLM | 10 | 30 | 8 | 2,8 | 3 |
| PM | 40 | NS ¹ | 3 | 14 | 22 |
| PC deficiency | NS | 26 | 7 | 0 | 0 |
| PS deficiency | NS | 43 | 2 | 30 | 2 |
| AT deficiency | NS | 26 | 1 | 4,5 | 3 |

All values except number of patients (*n*) indicate percentage of patients.

¹NS, not studied.

disorders (2-28 %), but in nearly 50% of the patients etiology remained unidentified^[28-32]. But with the advent of better medical care, potent antibiotic treatment, advanced medical technology, discovery of various causes of genetic thrombophilia and with better understanding of the coagulation system, this profile has also changed.

Identification of etiology usually starts by exclusion of local factors such as cirrhosis, primary or metastatic cancer of liver, pylephlebitis, liver cysts, vascular abnormalities (like webs or aneurysms) and pancreatitis. Besides routine investigations such as liver biopsies or Doppler-USG studies, magnetic resonance or CT based (3 dimensional angiographic images also available) techniques are very helpful at this stage. If no local lesion is found, then the studies are directed for a possible thrombophilic condition. After all, if no factor can be found, then the condition should be named as "idiopathic PVT".

In a study by Denninger *et al*^[33], the underlying prothrombotic condition was identified in 72 % of PVT patients. There was one or more prothrombotic condition in 26 out of 36 patients studied and primary myeloproliferative disorders were the leading cause with a prevalence of 30 % in this study. Other studies by Valla *et al*^[34-36] and De Stefano *et al*^[37] report the similar figure that as a cause of PVT, myeloproliferative disorders (overt or occult) rank first in the thrombophilic conditions. Valla *et al* also proposed that overt or latent myeloproliferative disorders may be responsible for thrombophilic milieu for PVT in 48% of patients.

The importance and impact of genetic thrombophilic factors in PVT are investigated in various studies. Table 2 shows the recent and most comprehensive studies about this topic published between 1999-2005. One of the most interesting findings about the results of these studies is the differences between them. Although all of the studies investigated the same patient population, the results are totally different from each other. The explanations for this situation may be the patient selection bias which is the problem of the tertiary care facilities, small number of patients, non-standard evaluation of the same parameters and genetic differences of the patient population.

The investigation of PC, PS and AT deficiencies in patients with PVT is a challenge for the clinician to interpret when the results are found to be lower than normal. There are studies investigating whether the low values are result of liver dysfunction or indicate a frank deficiency. One

of the most important studies is performed in children. Mack *et al*^[42] studied the coagulation parameters (including factors synthesized exclusively in liver) in 11 children with PVT who underwent a surgical correction (mesenterico-left portal vein bypass: Rex Shunt) of the portal venous system before and after surgery. The investigators found a significant correction of both coagulation factors (factors II, V and VII) indicated also by improvement in prothrombin time and of PC and PS levels after surgery. The same parameters studied in other 2 children receiving distal splenorenal shunt in this study and 7 children in another study receiving H-type mesocaval shunt^[43] did not improve as the Rex shunting supporting the importance of lower portal vein flow in the development of secondary PC and PS deficiencies. Fisher *et al*^[44] studied the same topic by combining the family investigation of PC and PS deficiencies in patients with PVT. They found out that there were only 3 familial cases of natural anticoagulant deficiencies out of 18 patients indicating a secondary finding. Another support to this finding and the previous results is that they have found significant reductions in AT, PC and PS concentrations after distal splenorenal shunt surgery (3 patients). All of the mentioned studies confirm that the lower portal blood flow has a great impact over the synthesis of natural anticoagulant and coagulant proteins. The shunt procedures directing the blood from portal system towards systemic circulation may worsen this as evidenced from comparison of results of splenorenal shunt vs. Rex shunt. In our opinion this phenomenon of secondary loss of coagulant and anti-coagulant proteins must be considered carefully in clinical practice when anticoagulation with warfarin is considered.

Secondary deficiencies of genetic thrombophilia factors like AT, PC and PS is still a matter of diagnostic challenge. To overcome this problem, there seems to be a very narrow range of solutions such as family study (first degree relatives) of deficient proteins in an attempt to detect familial aggregation of mutant genes and primary genetic studies of the patient to detect the mutant gene(s). These studies are important but not practical and not applicable in every facility. A practical screening method for detection of natural anticoagulant deficiencies in patients with liver disease was proposed by Pabinger *et al*^[45] and used firstly by Janssen *et al*^[39] in a clinical study which utilizes the ratio of natural anti-coagulant factor to coagulation factors (synthesized exclusively in liver). The formula is the ratio of PS or PC or AT (whichever tested) to [(factor II + factor X) / 2]. If the result of this ratio is lower than 70 %, then this may indicate a deficiency of the tested natural anti-coagulant protein significantly disproportionate to decreased synthesis of proteins from liver. This formula is easy to use in clinics and is practical to determine a possible genetic deficiency.

The concept of PVT can not be solely attributed to one factor. It is now widely accepted that the PVT occurs from both a primary thrombophilic milieu and a factor that triggers the formation of the pathological thrombus in portal circulation. The aforementioned factors are currently considered as thrombophilic background rather than the primary etiologic factor according to the concept of multifactorial theory of thrombogenesis. The other co-

factors required to develop a thrombus can be grouped as local and systemic factors most of which are potentially curable or preventable factors.

Clinical consequences

The consequences of the PVT vary from one patient to another and depend on some important factors. The condition may present itself as one of the complications but due to lack of convincing evidence in the literature, the most common type of initial presentation of PVT (not related with cirrhosis) is still not known. In our personal experience, most common type of presentation is variceal bleeding followed by pancytopenia due to hypersplenism. Undefined cholestasis related with pseudocholangiocarcinoma sign (without icterus) is also one of the rare presentations.

With the advent and widely distribution of USG and Doppler-USG, the condition is becoming to be diagnosed very earlier and a patient presenting with ascites (which is a very late finding in course of PVT) is almost not seen. Webb and Sherlock^[27] reported in 1979 that out of 97 patients with PVT 13 presented with ascites which seems to be a very high rate (13.5%). There are still some PVT cases presenting as ascites in underdeveloped parts of the world related with absence of early diagnosis with similar rates around 13%^[46].

Varices Esophageal and gastric varices related bleeding contribute to the most important cause of morbidity and hospitalization in this group of patients. When the characteristics of variceal bleeding are considered, major differences between cirrhosis *vs* PVT related bleeding must be pointed out. Firstly, the risk of variceal bleeding in cirrhosis is approximately 80-120 times more than PVT (non-cirrhotic) related variceal bleeding^[47-49] and esophageal varices (irrespective of size) are observed in about 90% of patients. Gastric varices are mostly seen concomitantly with esophageal varices in about 40% of the patients in PVT while portal gastropathy is also a rare feature of this condition^[49, 50]. Compared to portosystemic varices observed in cirrhotic patients, the varices in PVT patients have some special characteristics: (1) “varice-on-varice” finding is less commonly observed; (2) the sizes of the varices are smaller; (3) associated portal gastropathy is less commonly present^[50]. The retrospective study (investigated the determinants of survival in a heterogenous group of patients including 124 non-cirrhotic *vs* 48 cirrhotic PVT cases) of Janssen *et al*^[47] showed that either the presence of varices or bleeding episodes of varices had no impact on survival rates and the most common cause of death was malignancy related causes. Twenty four percent of the study population had malignancies like hepatocellular cancer, pancreatic cancer, or other malignancy elsewhere metastatic to the liver which presented itself in liver as PVT indicating end stage malignancies.

Although the risk of variceal bleeding is low in non-cirrhotic patients, the management of bleeding varices is not different from cirrhotic patients. Unfortunately there is not any study in the literature comparing endoscopic procedures *vs* surgical procedures in acutely bleeding varices and about the topic of primary prophylaxis. We believe that primary prophylaxis with endoscopic procedures in



Figure 3 ERCP of a patient with portal cavernomatous transformation. Arrow shows the site of depression in the main bile duct.

an effort to clear varices should be applied with careful monitoring and secondary prophylaxis with combination of endoscopic procedures and medical treatment despite beta-blockers may work although there is no objective and sufficient evidence.

In contrast, beta-blockers may result in more sluggish portal flow as is the same issue for nitrates and terlipressin increasing the risk of progression of thrombosis and even worsening of portal hypertension. This decision must be adjusted with the patient's condition rather than a standard medical care.

Pseudocholangiocarcinoma sign One of the interesting and misleading clinical conditions that may occur during the course of the PVT is “pseudocholangiocarcinoma sign” (PSCS). After the obstruction in the portal system, the “venous rescue” begins to occur immediately which takes about 5 wk^[24] forming new vessels around intrahepatic, extrahepatic biliary tracts and around gallbladder (majorly, vascular plexus of Saint and Petren enlarge and dilate to become large serpentine vessels) named as “portal cavernomatous transformation”. Sometimes these vessels can be very small in caliber to visualize depending on the extent of portal flow and capacity of new vessel formation, but if a careful ERCP is performed (Figure 3) the direct effects of these vessels on biliary ducts or main bile duct as strictures, displacements, thumbprinting effects or irregularity can be seen in at least 80% of the patients^[49]. This condition mimics a cholangiocellular cancer in ERCP and therefore called PSCS^[51, 52].

The clinical impact of these changes results in a wide spectrum of findings ranging from totally normal biochemical and physical findings to overt cholestasis with elevated cholestatic enzymes and liver injury (very rarely observed). Although a term called “portal biliopathy” and a classification system are proposed^[53], we believe that this term and classification system are impractical to use because: (1) new collaterals form depending on the site and level of portal vein obstruction, (2) wide range of anatomical variations between human beings in this anatomical site resulting in different types of cavernous transformations, (3) the nature of new vessel formation is unpredictable and not standard in all portal vein thrombosis patients, (4) the classification system does not have impact over treatment or follow up strategy. We propose that only defining the presence or absence of PSCS is a satisfactory practice not to complicate the picture further because the clinically evident cholestasis and/jaundice is observed in only about

5%^[54]. Therefore this physiological compensatory response is not a “-pathy” but just an adaptive change with a low degree of clinical importance.

The clinically evident cholestasis either presenting itself as repeating biliary stones or cholangitis or liver injury can be treated with conventional methods including stenting, papillotomy or even surgery.

3Hypersplenism and issue of anticoagulation treatment Hypersplenism is a finding of latent or chronic PVT and this complication is important when anticoagulation treatment is considered. Hypersplenism is almost always present except in the rare patient with a partial thrombus in one branch of the portal vein with the other branch remaining intact presenting with a mild degree of portal hypertension. The levels of all blood elements begin to decrease as the condition worsens and severe hypersplenism is now considered one of the most important indications in this group of patients to undergo a shunt surgery combined with or without a splenectomy. In the clinical setting of hypersplenism with low platelet counts combined with esophageal varices it raises concerns about the safety of anticoagulation. Unfortunately, the literature lacks information about the safety and long-term results of anticoagulation in well designed prospective controlled studies. A study to clarify this controversial topic was a retrospective analysis. In this study Condat *et al*^[48] included 136 PVT patients in whom 84 received anticoagulant treatment. The study has some limitations like heterogenous groups (pre-treatment endoscopies of all patients and standardization of a homogenous varice distribution between two groups were not accomplished) and lack of information about comorbid conditions of the participating patients, but the study has a low statistical degree of evidence to favor anticoagulation treatment. Anticoagulation was not found to be a risk factor for bleeding in this study while non-anticoagulation resulted in more thrombotic recurrences as expected. In our personal experience it may be a good practice to select patients according to their co-morbid conditions, degree of hypersplenism (low platelet counts may be a relative contraindication for anticoagulation) and the condition of varices (eradication combined with or without medical prophylaxis before start of treatment may be considered to decrease bleeding related risks).

BUDD-CHIARI SYNDROME: ETIOLOGY, CLINICAL CONSEQUENCES

Budd-Chiari syndrome (BCS) is a disorder which (besides its rarity and heterogenous clinical presentations) can potentially result in mortality and severe morbidities including liver failure requiring transplantation and even hepatocellular cancer. Venous-occlusive liver disease (also defined as sinusoidal obstruction syndrome) is a different pathology and therefore not included in this discussion.

There are various classifications of BCS in the literature. Classification systems include; 1) differences of etiology (primary: related with thrombophilia and secondary: due to tumor or a mass occupying lesion), 2) differences of anatomical involvement (site of obstruction: small and/or large hepatic veins, isolated inferior vena cava involvement, or combination of all), 3) progression

of disease (acute/fulminant and chronic/indolent). The forthcoming discussion about BCS involves primary BCS unless otherwise specified.

Etiology

Major underlying mechanism resulting in BCS is thrombosis. The etiology of thrombosis is very similar to PVT as discussed earlier, myeloproliferative disorders are the leading cause in 20-50% of all cases which may be overt or latent in presentation^[33,39]. The latent myeloproliferative disorders (for instance; endogenous erythroid colonies) are not easily detectable and requires an expert laboratory and sensitive tests (bone marrow is cultured *in vitro* to observe whether there is spontaneous erythroid colonies formed in the absence of erythropoietin stimulation). The mirror image of relation between myeloproliferative diseases and BCS is also interesting. In an autopsy study by Wanless *et al*^[55], the hepatic vein thrombosis was found in 6% of all subjects having a history of myeloproliferative disease. Paroxysmal nocturnal hemoglobinuria is also an acquired thrombophilic disorder characterized by occasional hemoglobinuria, venous thrombosis and anemia. It poses a high risk for development of BCS, about up to 10 % of all paroxysmal nocturnal hemoglobinuria patients have thrombosed hepatic veins^[56].

Behcet's disease is a subtype of large vessel vasculitis observed mostly in Turkish population which is related with hepatic vein thrombosis very frequently. Behcet's disease is associated with recurrent oral and genital ulcers with skin findings (erythema nodosum, pathergy reaction), neurologic involvement and eye inflammation. BCS occurring in Behcet's disease must be treated with an appropriate combination of colchicine, thalidomide, penicillins and anti-coagulating agents^[20,57,58].

Pregnancy and use of oral contraceptives are also related with BCS but studies in the literature have conflicting results due to study designs and the number of subjects included. The studies will not be discussed in detail but the exact relation of both conditions must be studied with adequate number of patients, defining other concurrent acquired and inherited thrombophilic conditions, estrogen content of the oral contraceptive studied. Nevertheless, both of these conditions have a theoretical risk for BCS.

Inherited causes of thrombophilia like FVLM, AT, PC and PS deficiencies account for a considerable percent age in the list of etiology of BCS. FVLM is found to be associated with 25-30% of all cases^[59,60], whereas PC deficiency (accounts for 9-20%) leads in all natural anticoagulant deficiencies^[33,39].

Other acquired causes of BCS include oral contraceptive use, pregnancy, Behcet's disease and inflammatory bowel disease.

Clinical consequences

The obstructed hepatic veins present to clinic in variable forms. The most common findings are ascites, hepatomegaly, pain in the abdomen and less frequently jaundice. Although the clinical findings are more florid with drastic consequences in fulminant forms, indolent



Figure 4 An MRI venography of hepatic veins and inferior vena cava. Arrow shows the site of obstruction in vena cava and invisible hepatic veins.

BCS is an insidious process in which development of findings mimic chronic liver disease of any other kind. The latter course results in development of portal hypertension and eventually cirrhosis combined with classical complications like variceal bleeding, intractable ascites and hypersplenism. Indolent course of BCS is characterized by pathological changes involving a variety of histological patterns like veno-portal fibrosis, veno-centric cirrhosis and nodular regenerative hyperplasia.

The studies describing the pathological changes unique to liver in chronic BCS have found that 1) the liver has an evolving change in terms of vascular dynamics to eventually result in changes in portal system (either intrahepatic or extrahepatic portal system becomes affected in time which shows itself as micro- or macro-thrombosis), 2) the liver is heterogenous, and there is great variability in random sampling (indicating that the liver biopsy must be multiple in number to clearly define the pathology and histological stage in BCS), 3) the regenerative hyperplasia with nodule formation of changing sizes are common which represents the areas with increased arterial supply with a patent venous drainage^[61,62].

Portal venous system must always be kept in mind when clinical presentation and management is considered. Portal system is almost always affected in patients with BCS. This results from various reasons: 1) perfusion of the portal system has a profound decrease since the sinusoidal pressure abruptly increases after initiation of BCS, 2) the enlarged and hypertrophied caudate lobe in response to BCS has a mass effect over main portal branches and intrahepatic portal venules resulting in stasis and vulnerability to thrombosis and further depleting liver perfusion. In patients with chronic BCS, the portal system is found to be involved in nearly 50% of patients when adequately studied either confined to the liver or as PVT.

The diagnosis of BCS can be made by utilizing Doppler-USG with a sensitivity of more than 85% in most cases^[63]. We believe that when combined with clinical presentation, Doppler-USG is readily the diagnostic tool of option when compared with CT or MRI techniques. One option that can be applied is directly having a non-invasive venography with CT or MRI (Figure 4) to clear out lesions in inferior vena cava, hepatic veins and portal veins. But this is expensive and not widely available. The portal vein involvement must always be investigated during initial evaluation for further follow-up and management decisions

since portal vein thrombosis has been shown to be a poor prognostic finding^[64]. The liver biopsy must also be a part of routine evaluation of BCS since it may be related with concomitant liver diseases, but there is no consensus in literature for the definition of an adequate biopsy in BCS. We believe there must be multiple biopsies from both lobes to clearly define, diagnose and estimate prognosis of BCS due to heterogenous nature of liver involvement.

Chronic liver disease eventually develops in nearly all BCS patients depending on the severity of liver involvement, potential of collateral development, co-morbid conditions (especially renal involvement, congestive or ischemic heart disease), thrombosis of other organs, progression of thrombosis in liver. Independent factors predicting survival include Child-Pugh score, age of the patient (younger age predicts better prognosis), renal functions and presence of portal vein involvement.

Management of the patients is directed towards prevention of recurrence of thrombosis and activate the natural thrombolytic potential (anticoagulation and antiplatelet treatment), treatment of complications of liver dysfunction (ascites, infections, renal failure) and relief of hepatic venous obstruction. In this sense, medical treatment has been proved efficacious in patients with an indolent, non-progressive (both histologically and biochemically) course^[65,66]. The repeated biopsies (in every 4-5 years) combined with biochemical follow-up usually suffices for this purpose. Doppler USG also must be a part of controls for both hepatic and portal venous system evaluations. The risk of anticoagulation and potential bleeding elsewhere including esophageal varices will not be discussed in detail. But we recommend that anticoagulation must be tailored for every patient according to the presence and stage of varices, history of previous bleeding, presence of thrombophilic condition and presence of portal vein thrombosis.

A failing liver with signs of decompensation indicated by refractory ascites, deterioration of Child-Pugh scores and renal failure preventing adequate medical therapy is definite and urgent indications for the need of relief of hepatic venous obstruction by means of vascular interventions including angioplasty, transjugular intrahepatic portosystemic shunt (TIPS) or surgery. TIPS and hepatic vein angioplasty are cost-effective techniques with a potential risk of recurrence of thrombosis^[67,68]. The effectiveness of vascular interventions has decreased the need for a shunt surgery which is rarely performed now. In selected patients with refractory, progressive disease despite adequate medical therapy and vascular interventions liver transplantation should be an option for absolute treatment. Sometimes presence of portal venous thrombosis itself precludes an angiographic intervention and results in need for a liver transplantation.

Liver transplantation performed for BCS has a five-year survival rate as high as 95%^[69]. Complications of transplantation are recurrence of thrombosis and development of secondary malignancies due to long-term immunosuppression. The presence of a myeloproliferative disorder underlying the BCS is not accepted as a contraindication for transplantation^[70].

MESENTERIC VEIN THROMBOSIS: ETIOLOGY, CLINICAL CONSEQUENCES

Mesenteric vein thrombosis (MVT) is an acute thrombotic disorder of vessels before formation of main portal vein. Some authors proposed to use the time-dependent definition for MVT, namely acute, subacute and chronic. We believe this classification should be abandoned and the term "MVT" be used for only acute venous obstruction of superior and/or inferior mesenteric veins, whereas chronic MVT itself is already PVT. Subacute thrombosis is an intermediary form of thrombosis in which the patient has abdominal pain and other intestinal symptoms. Therefore, the term MVT is used for acute mesenteric venous thrombosis in the rest of the discussion.

MVT presents itself mostly as a severe abdominal pain located mostly at periumbilical area with a blunt and colicky nature. It may be associated with nausea, vomiting, increased bowel movements and sometimes bloody diarrhea. With progression in time, the clinical picture converts itself into an acute abdomen with findings of rebound tenderness, fever, septicemia and finally a full blown peritonitis which is an inevitable consequence of erroneous diagnosis and treatment. Therefore, early diagnosis and rapid initiation of treatment is mandatory for a good clinical outcome.

Etiology

With the improving diagnostic tests in thrombophilia research, the number of idiopathic cases has decreased to 20 %. We believe this percentage will also decrease in time with advent of new techniques and development of new concepts of research. Currently the most important risk factors are considered as presence of a large spleen, cirrhosis, surgery, abdominal inflammation (pancreatitis, abscess, inflammatory bowel disease, diverticulitis), intraabdominal cancer, thrombophilic conditions (acquired or inherited). The importance of inherited thrombophilia is difficult to evaluate (sparing mutations like FVLM and PM) since the measurements of PC, PS and AT in the acute thrombosis stage and in the anti-coagulation treatment stage of the disease is not useful. The literature is lacking convincing evidence about the importance and prevalence of these disorders in the setting of MVT. One good example for this topic is the study by Kumar *et al*^[11]. In this study, the risk factors for MVT (comparing isolated MVT *vs* combined MVT with portosplenic venous thrombosis) are studied and they reported the inherited thrombophilic conditions (PC, PS and AT deficiencies, FVLM) in 27% and 5% of isolated MVT and combined MVT with portosplenic thrombosis, respectively. Interestingly, they found out that the risk of inherited thrombophilia was lower in patients with more extensive vessel involvement. Although the method and criteria of diagnosis of deficiencies of natural anticoagulants are not explained in detail due to retrospective design, this study is important to point the etiology and clinical characteristics of MVT in detail.

Although myeloproliferative disorders play a great role both in PVT and BCS, it does not seem to play the same role in case of MVT. This difference in presentation

between MVT and PVT has important clinical implications for further investigations.

Consequences

The mortality rate of MVT is about 20-50%, increasing in parallel to the older age and presence of comorbid clinical conditions (cirrhosis, cardiac failure, etc.). We believe the most important factor to determine the morbidity and mortality is the prompt diagnosis and rapid initiation of treatment. Clinical suspicion has utmost importance in early diagnosis due to the obscure nature of the initial clinical presentation. History of venous thrombosis, older age, use of oral contraceptives, presence of other thrombophilic conditions are indications to rule out a possible MVT. Although the gold standard diagnostic modality is a conventional angiography, CT establishes diagnosis in nearly 90 % of patients^[7,2,73].

After diagnosis the treatment must be initiated with anti-coagulation unless the patient has peritoneal irritation findings in which surgery is indicated. The most extensive analysis including 45 studies (a total of 3692 patients) about survival in mesenteric ischemia was performed by Schoots *et al*^[74]. They found that MVT has a better survival (including study populations requiring surgical resection of bowel and conservative treatment groups) compared to arterial or non-thrombotic ischemia of the bowel. This finding seems to be related with a limited involvement of bowel in MVT.

CONCLUSION

Thrombophilia has a wide range of clinical presentations but abdominal organs like liver and intestines bear the most important and interesting sequelae. The consequences of thrombophilia like PVT, BCS and MVT are potentially treatable if only diagnosed early and prompt treatments are initiated. With the advent of new technologies in research of thrombophilia, clinicians will be able to diagnose the idiopathic thrombosis group of patients which is a topic of controversy. Although anticoagulant treatment is mandatory, some limitations and contraindications may prevent their use. Lastly, these group of patients must be followed with a consulting surgeon who has experience in this field to decide the best timing for a shunt surgery and for possible acute operation indications.

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Serum tumor markers for detection of hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is one of the most frequent malignant tumors and is the second most common cause of cancer death in China. Therefore, it is very important to detect this disease and the recurrence at its earlier period. Serum tumor markers, as the effective method for detecting hepatocellular carcinoma for a long time, could be divided into 4 categories: oncofetal antigens and glycoprotein antigens; enzymes and isoenzymes; genes; and cytokines. Serum alpha fetoprotein (AFP) is the most widely used tumor marker in detecting patients with hepatocellular carcinoma, and has been proven to have capability of prefiguring the prognosis. However, it has been indicated that AFP-L3 and DCP excel AFP in differentiating hepatocellular carcinoma from nonmalignant hepatopathy and detecting small hepatocellular carcinoma. Some tumor markers, such as human cervical cancer oncogene and human telomerase reverse transcriptase mRNA, have also been indicated to have higher accuracies than AFP. Furthermore, some other tumor markers, such as glypican-3, gamma-glutamyl transferase II, alpha-L-fucosidase, transforming growth factor-beta1, tumor-specific growth factor, have been indicated to be available supplementaries to AFP in the detection. AFP mRNA has been shown to correlate with the metastasis and recurrence of HCC, and it may be the most useful marker to prefigure the prognosis. Some other markers, such as gamma-glutamyl transferase mRNA, vascular endothelial growth factor, and interleukin-8, could also be used as available prognostic indicators, and the simultaneous determination of AFP and these markers may detect the recurrence of HCC at its earlier period.

Key words: Hepatocellular carcinoma; Serum tumor markers; Sensitivity; Specificity; Prognosis

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INTRODUCTION

Hepatocellular carcinoma (HCC), one of the most frequent malignant tumors, is the second common cause of cancer death in China, where its mortality rate is 20.37/100 thousand^[1]. Surgical resection is the most effective method for curing this disease, but a large amount of cases are not adapted to surgery because of their intrahepatic or distant metastases at the time of diagnosis. Furthermore, the long-term survival of postoperative HCC patients is unsatisfactory for the high incidence of recurrence. Therefore, it is very important to detect HCC and the recurrence at its earlier period. By reasons of convenience, inexpensiveness, and the satisfactory accuracy, serum tumor markers have been used as an effective method for detecting malignant tumors for a long time, and they could be valuable supplementaries to ultrasonography and computer tomography in the diagnosis of HCC. Using the appropriate single or combination of tumor markers may improve the effectiveness in screening HCC patients.

ONCOFETAL ANTIGENS AND GLYCOPROTEIN ANTIGENS

Alpha-fetoprotein and alpha-fetoprotein-L3

Alpha fetoprotein (AFP) is a fetal specific glycoprotein produced primarily by the fetal liver. Normally, its serum concentration falls rapidly after birth and its synthesis in adult life is repressed. However, greater than 70% of HCC patients have a high serum concentration of AFP because of the tumor excretion. Forty years after its discovery, serum AFP remains a most useful tumor marker in screening HCC patients. The serum concentration of 20 ng/mL is the most commonly used cut-off value to differentiate HCC patients from healthy adults in clinical researches. However, some investigations have showed that the cut-off value is fluctuant in different ethnic groups.

The best cut-off value of AFP has been reported to be 30 ng/mL (sensitivity of 65%, specificity of 89%) in Sicilian population compared with 200 ng/mL (sensitivity of 70%, specificity of 100%) in Burman population^[2,3]. One of possible reasons for this difference is the diverse living circumstance which has a great influence on epidemiology. Besides the purpose of screening HCC, serum and tissues AFP could also be used as prognostic indicators^[4]. HCC patients with a high AFP concentration (≥ 400 ng/mL) tend to have greater tumor size, bilobar involvement, massive or diffuse types, portal vein thrombosis, and a lower median survival rate^[5,6]. This is partially caused by the expression of ephrin-A1 (an angiogenic factor) and the ability of AFP to elicit the escape of carcinoma cells from the host's lymphocytes immune surveillance^[7,8]. Though the measurement of AFP serves as an important tool in screening HCC patients, some reports have indicated that it has limited utility of differentiating HCC from benign hepatic disorders for its high false-positive and false-negative rates, and patients with acute exacerbation of viral hepatitis but no HCC may also have markedly increased AFP levels^[9]. Using the cut-off value of 20 ng/mL to differentiate HCC from HCV-infected patients, sensitivities merely range from 41% to 65% with specificities of 80% to 94% correspondingly^[10]. Moreover, the positive predictive value (PPV) of AFP is significantly lower in detecting HCC patients with viral etiology than that in detecting HCC patients with non-viral etiology (70% *vs* 94%, $P < 0.05$), and it will not reach 100% in HCC patients with viral etiology unless their serum concentration of AFP is greater than 400 ng/mL^[2,11]. Therefore, AFP is more useful in detecting HCC patients with non-viral etiology.

Total AFP can be divided into three different glycoforms, namely AFP-L1, AFP-L2 and AFP-L3, according to their binding capability to lectin lens culinaris agglutinin (LCA). AFP-L1, as the non-LCA-bound fraction, is the major glycoform of AFP in the serum of nonmalignant hepatopathy patients. On the contrary, AFP-L3, as the LCA-bound fraction, is the major glycoform of AFP in the serum of HCC patients, and it can be detected in approximately 35% of patients with small HCC (< 3 cm), especially when the tumor mass is supplied by the hepatic artery. At the cut-off level of 15%, sensitivities of AFP-L3 in detecting HCC range from 75% to 96.9% with specificities of 90% to 92.0% correspondingly^[3,12]. Furthermore, some clinical researches have indicated that the high percentage of AFP-L3 is closely related to poor differentiation and biologically malignant characteristics (especially portal vein invasion) of HCC^[12,13], and HCC patients with positive AFP-L3 would have worse liver function, poorer tumor histology, and larger tumor mass^[14]. Compared with those with serum concentration of des-gamma-carboxyprothrombin (DCP) over 100 mAU/mL, HCC patients with percentage of serum AFP-L3 over 15% also showed a higher incidence of infiltrative-type HCC with an irregular margin ($P < 0.05$) and a higher frequency of poorly differentiated HCC ($P < 0.01$)^[15]. Therefore, it could be used as a valuable indicator of poor prognosis.

Glypican-3

Glypican-3 (GPC3) is a heparan sulfate proteoglycan anchored to the plasma membrane. It has been demonstrated to interact with growth factors and modulate their activities. The expression of GPC3 (at both mRNA and protein levels) in the serum of HCC patients is significantly higher than that in the serum of healthy adults ($P < 0.001$) or patients with nonmalignant hepatopathy ($P < 0.01$), and it can be detected in 40-53% of HCC patients and 33% of HCC patients with seronegative for both AFP and DCP^[16-18]. Some clinical researches have indicated that the simultaneous determination of GPC3 and AFP could significantly increase the sensitivity in the diagnosis of HCC^[16]. Furthermore, it has been shown that soluble GPC3 (sGPC3), the NH2-terminal portion of GPC3, is superior to AFP in the sensitivity of detecting well or moderately differentiated HCC, and the simultaneous determination of both markers improves overall sensitivity from 50% to 72%^[17]. Thus, it can be seen that GPC3 could be a good supplementary to AFP in the detection. Some other investigators have reported that GPC3 mRNA is upregulated significantly in tumor tissues of HCC compared to paraneoplastic tissues of HCC, liver tissues of healthy adults and liver tissues of patients with nonmalignant hepatopathy, thus it could also be a good molecular marker for HCC^[16,19-21].

ENZYMES AND ISOENZYMES

Gamma-glutamyl transferase

Serum gamma-glutamyl transferase (GGT) in healthy adults is mainly secreted by hepatic Kupffer cell and endothelial cell of bile duct, and its activity increases obviously in tissues of HCC and fetal liver. Total GGT can be divided into 13 isoenzymes (I, I', II, II', β , δ , ϵ , φ A, VIII, φ C, γ A, γ B) by using polymeracrylamide gradient gel electrophoresis, and some of them (I', II, II') can only be detected in the serum of HCC patients. Sensitivities of GGII have been reported to be 74.0% in detecting HCC and 43.8% in detecting small HCC^[22]. Furthermore, the simultaneous determination of GGII, DCP, and AFP can significantly improve the sensitivity over AFP alone^[22]. It should be a valuable tumor marker in detecting small HCC and a good supplementary to AFP in the diagnosis of HCC.

Alpha-L-fucosidase

Alpha-L-fucosidase (AFU) is a sort of enzyme to hydrolyze fucose glycosidic linkages of glycoprotein and glycolipids. Its activity increases obviously in the serum of HCC patients ($1\ 418.62 \pm 575.76$ nmol/mL per hour) compared with that in the serum of healthy adults (504.18 ± 121.88 nmol/mL per hour, $P < 0.05$), patients with cirrhosis (831.25 ± 261.13 nmol/mL per hour), and patients with chronic hepatitis (717.71 ± 205.86 nmol/mL per hour)^[23,24]. It has been reported that the sensitivity and specificity of AFU at the cut-off value of 870 nmol/(mL per h) are 81.7% and 70.7%, respectively, in contrast with 39.1% and 99.3% of AFP at the cut-off value of 400 ng/mL, and the

simultaneous determination of both markers can improve the sensitivity to 82.6%^[23]. This indicates that AFU could serve as a valuable supplementary to AFP in the detection. Furthermore, it has been indicated that HCC will develop within a few years in 82% of patients with liver cirrhosis, if their serum AFU activity exceeds 700 nmol/(mL per h), and the activity of AFU is already elevated in 85% of patients at least 6 months before the detection of HCC by ultrasonography^[24]. Thus, it can be seen that AFU could be a good tumor marker in detecting HCC at the earlier period.

Des-gamma-carboxyprothrombin

DCP, also known as a protein induced by vitamin K absence or antagonist II (PIVKA-II), is an abnormal product from liver carboxylation disturbance during the formation of thrombogen, and acts as an autologous mitogen for HCC cell lines^[25]. Its mean serum concentration, which is not correlated to serum levels of AFP, is obviously elevated in HCC patients compared with that in healthy adults and patients with nonmalignant hepatopathy^[26,27]. Though a few researches have an opposite result^[28], serum and tissues DCP have been proved to be more useful than AFP in differentiating HCC from nonmalignant hepatopathy and in detecting patients with small HCC^[6,26,27,29]. Cui *et al.*^[26] reported that the sensitivity and specificity of serum DCP (at the most commonly used cut-off value of 40 mAU/mL) in discriminating HCC from cirrhosis were 51.7% and 86.7%, respectively, which were much better than those of AFP at the cut-off value of 20 ng/mL, and 36.84% of patients with small HCC had serum DCP values above this level. Marrero *et al.*^[27] reported that the sensitivity and specificity of serum DCP (at the cut-off value of 125 mAU/mL) in discriminating HCC from nonmalignant hepatopathy were 89% and 86.7%, respectively, which were much better than those of AFP at the cut-off value of 11 ng/mL. Furthermore, the simultaneous determination of DCP and other tumor markers, such as AFP and AFP-L3, may have a greater accuracy than the determination of each of them alone^[26,30-32]. It has been reported that the electrochemiluminescence enables measurement of low-concentration of DCP (high-sensitive DCP) in the serum, and the simultaneous determination of high-sensitive DCP (at the cut-off value of 40 mAU/mL), AFP (at the cut-off value of 20 ng/mL), and AFP-L3 (at the cut-off value of 10%) gives the highest accuracy (sensitivity of 82.1%, specificity of 82.4%, and accuracy of 82.2%)^[32]. Besides the purpose of screening HCC, serum DCP could also be used as a clinicopathological or prognostic indicator for HCC patients, and may be more useful than AFP in reflecting the invasive characteristics of HCC^[28,33,34]. It has been reported that patients with DCP seropositive and AFP seronegative have a higher frequency of HCC with a distinct margin, large nodule more than 3 cm, few nodules, and moderately to poorly differentiation^[33,34]. Moreover, the simultaneous determination of serum DCP levels and tissue DCP expression is more valuable than either factor alone in predicting the prognosis of HCC patients^[35].

GENES

Alpha-fetoprotein mRNA

Though a few researches have an opposite result^[36], HCC cells spread into blood circulation and become the source of recurrence after operation, which may be the primary reason for the unsatisfactory long-term survival after surgery, and the presence of circulating HCC cells may be indicative of metastasis if AFP mRNA is detected in peripheral blood^[37]. A large number of clinical researches have indicated that serum AFP mRNA detected by reverse-transcription polymerase chain reaction (RT-PCR) may be a valuable indicator of poor prognosis for HCC patients, and its expression is correlated with portal thrombosis, nodules of tumor, tumor diameter, and TNM stage ($P < 0.05$)^[38-42]. The recurrence-free interval of HCC patients with postoperative serum AFP mRNA positivity has been reported to be significantly shorter than that of HCC patients with postoperative negativity (53% *vs* 88% at 1 year, 37% *vs* 60% at 2 years, $P = 0.014$)^[42], and the recurrence-free survival rates of HCC patients with postoperative serum AFP mRNA positivity have been reported to be significantly lower than those of HCC patients with preoperative positivity (52.6% *vs* 81.8% at 1 year, 15.6% *vs* 54.5% at 2 years, and 0% *vs* 29.2% at 3 years, $P < 0.001$)^[43]. From the result of meta-analysis, the expression of AFP mRNA one week after surgery has also been showed to be correlated with the recurrence of HCC^[44]. Moreover, the simultaneous determination of AFP mRNA and melanoma antigen gene (MAGE-1) mRNA may have a higher sensitivity and specificity^[41].

Gamma-glutamyl transferase mRNA

GGT mRNA can be detected in the serum and liver tissues of healthy adults or patients with HCC, nonmalignant hepatopathy, hepatic benign tumor, and secondary carcinoma of liver. It can be divided into three types: fetal liver (type A), HepG2 cells (type B), and placenta (type C). Type A is predominant in normal liver tissues or liver tissues with nonmalignant hepatopathy, benign tumor, and secondary carcinoma ($P < 0.05$). On the contrary, type B is predominant in cancerous tissues of HCC ($P < 0.05$)^[45-48]. During the development of HCC, the expression of GGT mRNA in liver tissues may shift from type A to type B^[48]. It has been indicated that HCC patients with positive type B would have a worse outcome, earlier recurrence, and more post-recurrence death ($P = 0.0107$)^[49]. Therefore, the expression of tissues type B may be a valuable indicator of poor prognosis for HCC patients. The same as in liver tissues, the serum levels of type B have also been reported to be significantly higher in HCC patients than in healthy adults ($P < 0.05$)^[46]. Therefore, serum type B may be an available supplementary to AFP in the diagnosis of HCC.

Human telomerase reverse transcriptase mRNA

Human telomerase reverse transcriptase (hTERT) mRNA has been reported to be detectable in the serum of patients with breast cancer. Furthermore, it has also been demonstrated to be a novel and available marker for HCC diagnosis. The expression of hTERT mRNA in the serum

of HCC patients is significantly higher than that in the serum of healthy adults or patients with nonmalignant hepatopathy^[50, 51], and the use of newly developed real-time quantitative reverse transcription polymerase chain reaction may improve the effectiveness of determination^[50]. It has been reported that the sensitivity and specificity of hTERT mRNA in detecting HCC are 88.2% and 70.0%, respectively, which excel those of conventional tumor markers, such as AFP mRNA, AFP and DCP^[50]. Moreover, it has been indicated that the expression of serum hTERT mRNA, which is associated with the serum concentration of AFP, tumor size, and tumor differentiation degree ($P < 0.001$, each), may be a valuable indicator of poor prognosis for HCC patients^[50, 51].

There are some other markers in this category, which could be used as diagnostic or prognostic indicators for HCC. It has been reported that the simultaneous determination of p53 antigen and anti-p53 antibodies has a sensitivity of 41.1% in the diagnosis of HCC^[52], and the over-expression of p53 in the serum or liver tissues of HCC patients prefigures the poorer prognosis and a shorter survival time ($P = 0.0014$)^[52-56]. HCC patients with positive MAGE-1 or MAGE-3 mRNA die earlier because of metastasis or recurrence^[57]. The sensitivity and specificity of serum human cervical cancer oncogene (HCCR) at the cut-off value of 15 $\mu\text{g}/\text{mL}$ in detecting HCC are 78.2% and 95.7%, respectively. Moreover, its sensitivities could achieve 76.9% in detecting HCC patients with seronegative for AFP and 69.2% in detecting HCC patients with tumor size less than 2 cm^[58].

CYTOKINES

Vascular endothelial growth factor

Vascular endothelial growth factor (VEGF) is a secreted homodimeric cytokine that positively regulates tumor neovascularization^[59]. Recent researches have suggested that angiogenesis is essential in tumor growth and progression, including that of HCC, which are typically characterized by a high level of vascularization^[60-62]. In fact, it has been shown that the expressions of VEGF in cancerous tissues of HCC and HCC with microscopic venous invasion are significantly higher than that in normal liver tissues and HCC without microscopic venous invasion ($P < 0.05$), and HCC patients with over-expression of VEGF have a lower survival rate ($P < 0.05$)^[63, 64]. Platelets have been reported to act as transporters of tumor-originated VEGF. It has been indicated that serum VEGF per platelet count, as an indirect theoretical estimate of VEGF in platelets, in HCC patients is significantly higher than that in healthy adults and patients with nonmalignant hepatopathy ($P < 0.01$), and the high serum VEGF per platelet count ($> 1.4 \text{ pg}/10^6$) is associated with advanced stage of HCC, portal vein thrombosis, poor response to treatment, and shorter overall survival ($P < 0.01$)^[65]. Therefore, it may be an available diagnostic or prognostic indicator for HCC.

Interleukin-8

Interleukin-8 (IL-8) is a multifunctional CXC chemokine that affects human neutrophil functions, including

chemotaxis, enzyme release, and expression of surface adhesion molecules. It has direct effects on tumor and vascular endothelial cell proliferation, angiogenesis, and tumor migration. Recent researches have indicated that IL-8 regulates tumor cell growth and metastasis in liver^[66]. It has been reported that the preoperative serum IL-8 levels in HCC patients are significantly elevated compared with those in healthy adults (17.6 pg/mL vs 1.0 pg/mL , $P = 0.046$), and its high serum levels correlate with a large tumor size ($> 5 \text{ cm}$), absence of tumor capsule, presence of venous invasion, advanced pathological tumor-node-metastasis stage, and a poorer disease-free survival^[67]. Therefore, it may be an available diagnostic or prognostic indicator for HCC.

Transforming growth factor-beta 1

Transforming growth factor-beta1 (TGF- β 1) is a negative growth factor which correlates with cellular immunosuppression during the progression of HCC, and its serum levels in HCC patients have been shown to be obviously elevated compared with those in healthy adults and patients with nonmalignant hepatopathy ($P < 0.0001$)^[68, 69]. At the cut-off value of 800 pg/mL , the specificity of serum TGF- β 1 in detecting HCC has been reported to be over 95% which is similar to AFP at the cut-off value of 200 ng/mL , but the sensitivity of serum TGF- β 1 is 68% which excels AFP with the sensitivity of 24%^[68]. Moreover, the elevated serum TGF- β 1 can be detected in 23% of HCC patients with normal serum AFP values^[69]. These researches have indicated that TGF- β 1 may be a good supplementary to AFP in the diagnosis of HCC.

Tumor-specific growth factor

Malignant tumor can release tumor-specific growth factor (TSGF), which results in blood capillary amplification surrounding the tumor, into peripheral blood during its growing period. Therefore, the serum levels of TSGF can reflect the existence of tumor. It has been indicated that TSGF can be used as a diagnostic marker in detecting HCC, and its sensitivity can reach 82% at the cut-off value of 62 U/mL ^[70]. Furthermore, the simultaneous determination of TSGF and other tumor markers has been shown to give a higher accuracy. It has been reported that the simultaneous determinations of TSGF, AFP, CEA, TSA, and serum ferritin have a sensitivity of 97.5%^[70], and the simultaneous determinations of TSGF (at the cut-off value of 65 U/mL), AFP (at the cut-off value of 25 ng/mL) and serum ferritin (at the cut-off value of 240 $\mu\text{g}/\text{mL}$) have a sensitivity of 98.4% and specificity of 99%^[71].

There are some other markers, which could be used as diagnostic or prognostic indicators for HCC, in this category. It has been reported that the determination of serum insulin-like growth factor-II (IGF-II) (at the cut-off value of 4.1 mg/g , prealbumin) has a sensitivity of 63%, specificity of 90%, and accuracy of 70% in the diagnosis of small HCC. Moreover, the simultaneous determination of IGF-II and AFP (at the cut-off value of 50 ng/mL) can improve the sensitivity to 80% and accuracy to 88%^[72]. The over-expression of granulin-epithelin precursor (GEP) in cancerous tissues of HCC is associated with venous infiltration and early intrahepatic recurrence ($P < 0.05$)^[73].

CONCLUSION

Serum AFP is the most widely studied screening test for detecting HCC. The normal range for serum AFP levels is 10-20 ng/mL and a level >400 ng/mL is usually regarded as diagnostic. Furthermore, some reports have indicated that the high serum concentration of AFP correlates with the poor prognosis of HCC patients. However, two thirds of HCC patients with the nodule less than 4 cm have serum AFP levels less than 200 ng/mL and up to 20% HCC patients do not produce AFP^[74]. Moreover, it has limited utility of differentiating HCC from benign hepatic disorders for the high false-positive and false-negative rates. Serum AFP-L3 and DCP are also widely used as tumor markers for HCC, and have been indicated to be more valuable than AFP in differentiating HCC from nonmalignant hepatopathy, detecting small HCC, and predicting the prognosis. Considering the large population with cirrhosis and chronic hepatitis in our country, AFP-L3 and DCP may be more useful than AFP in the diagnosis of HCC. hTERT mRNA and HCCR have been shown to have a higher accuracy than AFP in detecting HCC, but there are not enough researches to manifest their superiority. Therefore, they may not be the first choice in the detection of HCC. IGF-II has been reported to be more valuable than AFP in the diagnosis of small HCC, however, more studies are needed to demonstrate its superiority. There are some serum markers, such as GPC3, GGT II, AFU, TGF- β 1, and TSGF, that have been indicated to be available supplementaries to AFP and DCP in the detection of HCC, and some of them even can be detected in HCC patients with seronegative for both AFP and DCP, the simultaneous determination of these markers may improve the accuracy. Serum AFP mRNA, which has been shown to be correlated with the metastasis and recurrence of HCC, may be the most useful marker to prefigure the prognosis of HCC patients. Some other markers, such as p53, MAGE-1, MAGE-3, GGT mRNA, VEGF, GEP, and IL-8, have also been indicated to be able to serve as prognostic indicators of HCC patients, the simultaneous determination of AFP and these markers may discover the recurrence of HCC at earlier period. In addition, there are some tumor markers, such as CYFRA 21-1^[75], activin-A^[76] and proliferating cell nuclear antigen^[54,77,78], which do not belong to each of the categories above, but they can also be used as prognostic or screening indicators for HCC patients, especially when combined with AFP.

In a word, AFP, AFP-L3 and DCP are the most useful serum tumor markers for the detection of HCC, and the simultaneous determination of these markers could improve the accuracy, especially in differentiating HCC from nonmalignant hepatopathy. Other tumor markers, which have been mentioned in our review, could be used as supplementaries to AFP and DCP in the diagnosis of HCC, but each of them has no satisfactory accuracy in detecting HCC or prefiguring the prognosis when used alone.

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GASTRIC CANCER

Prognostic factors in patients with node-negative gastric carcinoma: A comparison with node-positive gastric carcinoma

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Abstract

AIM: To identify the clinicopathological characteristics of lymph node-negative gastric carcinoma, and also to evaluate outcome indicators in the lymph node-negative patients.

METHODS: Of 2848 gastric carcinoma patients, 1524 (53.5%) were lymph node-negative. A statistical analysis was performed using the Cox model to estimate outcome indicators.

RESULTS: There was a significant difference in the recurrence rate between lymph node-negative and lymph node-positive patients (14.4% vs 41.0%, $P < 0.001$). The 5-year survival rate was significantly lower in lymph node-positive than in lymph node-negative patients (31.1% vs 77.4%, $P < 0.001$). Univariate analysis revealed that the following factors influenced the 5-year survival rate: patient age, tumor size, depth of invasion, tumor location, operative type, and tumor stage at initial diagnosis. The Cox proportional hazard regression model revealed that tumor size, serosal invasion, and curability were independent, statistically significant, prognostic indicators of lymph node-negative gastric carcinoma.

CONCLUSION: Lymph node-negative patients have a favorable outcome attributable to high curability, but the patients with relatively large tumors and serosal invasion have a poor prognosis. Curability is one of the most reliable predictors of long-term survival for lymph node-negative gastric carcinoma patients.

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Key words: Gastric carcinoma; Survival; Tumor size; Serosal invasion; Curability

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INTRODUCTION

The presence or absence of lymph node metastasis is one of the most important prognostic indicators among several clinicopathological factors that influence the prognosis of patients with gastric carcinoma^[1-4]. Several studies have been conducted to identify prognostic indicators in patients with lymph node-negative gastric carcinoma, but these studies have involved small numbers of patients^[5-9]. In the present study, we compared lymph node-negative patients with lymph node-positive ones to identify the clinicopathological characteristics of lymph node-negative gastric carcinoma. We also evaluated outcome indicators for lymph node-negative carcinoma.

MATERIALS AND METHODS

Patients and specimens

From 1986 to 2000, 2848 patients with gastric carcinoma were treated in the Division of Gastroenterologic Surgery, Department of Surgery, Chonnam National University Medical School, Gwangju, Korea. Of these, 1524 (53.5%) were in the lymph node-negative group.

The clinicopathologic features of these patients with lymph node-negative gastric carcinoma were retrospectively reviewed. Information on the patient's age, sex, tumor size, tumor location, macroscopic appearance, depth of invasion, hepatic metastasis, peritoneal dissemination, stage at the initial diagnosis, operative type, recurrence pattern, curability, and survival rate was obtained from the hospital records. The American Joint Committee on Cancer (AJCC) TNM Staging system was used for clinical and pathologic staging^[10]. Histological evaluation was performed according to the Japanese General Rules for Gastric Cancer Study in Surgery and Pathology^[11].

Statistical analysis

The survival rates of the patients were calculated using the Kaplan-Meier method and the relative prognostic

Table 1 Clinical features of patients with node-negative and -positive gastric carcinoma

| Variables | Node-negative (n = 1524) (%) | Node-positive (n = 1324) (%) | P value |
|---------------------------------|------------------------------|------------------------------|---------|
| Age (mean, yr) | 56.9±11.1 | 57.1±11.6 | NS |
| Gender | | | NS |
| Male | 988 (64.8) | 889 (67.1) | |
| Female | 536 (35.2) | 435 (32.9) | |
| Extent of lymph node dissection | | | NS |
| <D2 | 262 (17.2) | 246 (18.6) | |
| ≥D2 | 1,262 (82.8) | 1,078 (81.4) | |
| Operative type | | | <0.001 |
| Total gastrectomy | 251 (16.5) | 383 (28.9) | |
| Subtotal gastrectomy | 1,226 (80.4) | 902 (68.1) | |
| Proximal gastrectomy | 12 (0.8) | 3 (0.3) | |
| Others | 35 (2.3) | 36 (2.7) | |
| Curability | | | <0.001 |
| Curative | 1,492 (97.9) | 1,024 (77.3) | |
| Non-curative | 32 (2.1) | 300 (22.7) | |
| Recurrence | | | <0.001 |
| Locoregional | 29 (13.2) | 46 (8.5) | |
| Peritoneum | 163 (74.1) | 480 (88.4) | |
| Others | 28 (12.7) | 17 (3.1) | |

NS, not significant.

importance of the parameters was investigated using the Cox proportional hazards model. The χ^2 was used to evaluate the statistical significance of differences and *P* values less than 0.05 were considered statistically significant.

RESULTS

Table 1 summarizes the clinical findings in 1524 (53.5%) patients with lymph node-negative gastric carcinoma and 1324 (46.5%) patients with lymph node-positive gastric carcinoma. There was no significant difference in the mean age between lymph node-negative and lymph node-positive patients (56.9 *vs* 57.1 years, respectively). Of the 1524 patients with lymph node-negative gastric carcinoma, 988 (64.8%) were male and 536 (35.2%) were female. There were 889 males (67.1%) and 435 females (32.9%) in the group of 1324 lymph node-positive patients. Although there were more males than females in each group, the gender ratio was the same in both groups. Dissection above the D2 lymph node was performed in most patients in each group (82.8% and 81.4% of the lymph node-positive and lymph node-negative patients, respectively). Subtotal gastrectomy was the procedure performed most frequently (80.4%) in patients with lymph node-negative gastric carcinoma. The curative resection rate was significantly higher (97.9%, 1492/1534) in lymph node-negative patients than in node-positive patients (77.3%; 1,024/1,324; *P*<0.001), and the recurrence rate was significantly lower (14.4%) in lymph node-negative than in lymph node-positive patients (41.1%; *P*<0.001). Peritoneal recurrence was the predominant type of recurrence in both groups.

The histopathological features are listed in Table 2. The mean tumor size in patients with lymph node-negative

Table 2 Histopathologic features of node-negative and -positive gastric carcinoma

| Variables | Node-negative (n = 1524) (%) | Node-positive (n = 1324) (%) | P value |
|---------------------------|------------------------------|------------------------------|---------|
| Tumor size (mean, cm) | 2.9±2.0 | 5.0±2.7 | <0.001 |
| Location | | | NS |
| Upper | 123 (8.1) | 141 (10.7) | |
| Middle | 443 (29.0) | 358 (27.0) | |
| Lower | 946 (62.1) | 783 (59.1) | |
| Whole | 12 (0.8) | 42 (3.2) | |
| Macroscopic appearance | | | NS |
| EGC | | | |
| Elevated | 161 (20.0) | 27 (24.1) | |
| Depressed | 572 (71.2) | 69 (61.6) | |
| Flat | 71 (8.8) | 16 (14.3) | |
| Borrmann type | | | <0.001 |
| 1 | 47 (6.5) | 54 (4.5) | |
| 2 | 183 (25.4) | 218 (18.0) | |
| 3 | 441 (61.3) | 812 (67.0) | |
| 4 | 49 (6.8) | 128 (10.5) | |
| Depth of invasion | | | <0.001 |
| T1 | 804 (52.8) | 112 (8.5) | |
| T2 | 343 (22.5) | 170 (12.8) | |
| T3 | 338 (22.1) | 856 (64.7) | |
| T4 | 39 (2.6) | 186 (14.0) | |
| Hepatic metastasis | | | <0.001 |
| H (-) | 1,519 (99.7) | 1,269 (95.8) | |
| H (+) | 5 (0.3) | 55 (4.2) | |
| Peritoneal dissemination | | | <0.001 |
| P (-) | 1,509 (99.0) | 1,179 (89.0) | |
| P (+) | 15 (1.0) | 145 (11.0) | |
| Histologic type | | | <0.001 |
| Well-differentiated | 355 (23.3) | 165 (12.5) | |
| Moderately differentiated | 352 (23.1) | 339 (25.6) | |
| Poorly differentiated | 536 (35.1) | 642 (48.5) | |
| Signet ring cell | 189 (12.4) | 59 (4.5) | |
| Mucinous | 53 (3.5) | 95 (7.1) | |
| Others | 39 (2.6) | 24 (1.8) | |
| Stage | | | <0.001 |
| I | 1,145 (75.1) | 91 (6.9) | |
| II | 324 (21.3) | 124 (9.4) | |
| III | 30 (2.0) | 689 (52.0) | |
| IV | 25 (1.6) | 420 (31.7) | |

NS, not significant

gastric carcinoma (2.9 cm) was significantly smaller than that in lymph node-positive patients (5.0 cm; *P*<0.001). Most gastric carcinomas were located in the lower third of the stomach in both lymph node-negative (946 cases; 62.1%) and lymph node-positive patients (783 cases; 59.1%). There were no significant differences between the groups with respect to the locations of the carcinomas. An invasion depth limited to T2 was found more frequently in patients with lymph node-negative (75.3%) than in lymph node-positive patients (21.3%; *P*<0.001). Hepatic metastases were found in 0.3% of the lymph node-negative patients and in 4.2% of the lymph node-positive patients. Peritoneal dissemination was present in 1.0% of the lymph node-negative patients and in 11.0% of the lymph node-positive patients. There was a significant difference between the groups in the number of cases with

Table 3 Prognostic significance by univariate analysis of variables for patients with lymph node-negative gastric carcinoma

| Variables | 5-year survival (%) | P value |
|---------------------------------|---------------------|---------|
| Age (yr) | | <0.01 |
| <65 | 79.1 | |
| ≥65 | 70.7 | |
| Gender | | NS |
| Male | 74.4 | |
| Female | 81.3 | |
| Tumor size (cm) | | <0.001 |
| <2.0 | 88.5 | |
| 2 - 4.9 | 79.2 | |
| ≥5 | 53.6 | |
| Depth of invasion | | <0.001 |
| T1, T2 | 88.4 | |
| T3, T4 | 55.4 | |
| Location | | <0.001 |
| Upper third | 49.9 | |
| Middle third | 67.9 | |
| Lower third | 62.3 | |
| Histologic type | | NS |
| Differentiated | 78.4 | |
| Undifferentiated | 75.7 | |
| Operative type | | <0.001 |
| Total | 61.5 | |
| Subtotal | 80.9 | |
| Extent of lymph node dissection | | NS |
| <D2 | 67.6 | |
| ≥D2 | 77.3 | |
| Stage | | <0.001 |
| I | 88.4 | |
| II | 61.5 | |
| III | 17.9 | |
| IV | 22.3 | |

NS, not significant

hepatic metastases and peritoneal dissemination. Poorly differentiated adenocarcinoma was found more frequently in patients with lymph node-positive gastric carcinoma than in lymph node-negative patients (48.5% vs 35.1%, $P < 0.001$). Of the lymph node-negative patients, 1470 (96.4%) were classified as either stage I or II at the time of initial diagnosis.

The overall survival rate of the lymph node-negative patients (77.4%) was higher than that of the lymph node-positive patients (31.1%; $P < 0.001$) (Figure 1). The 5-year survival rate of patients with early lymph node-negative gastric carcinoma was significantly higher than that of patients with early lymph node-positive gastric carcinoma (93.3% vs 84.3%, $P = 0.0147$). Patients with advanced lymph node-negative gastric carcinoma also had a significantly higher 5-year survival rate than that of patients with advanced lymph node-positive gastric carcinoma (66.9% vs 33.1%, $P < 0.001$). The clinicopathological variables tested by univariate analysis are shown in Table 3. The factors that influenced the 5-year survival rate were patient's age, tumor size, depth of invasion, tumor location, operative type, and tumor stage at initial diagnosis. Using the Cox proportional hazard regression model, tumor size, presence of serosal invasion, and

Table 4 Multivariate analysis of significant prognostic factors for survival in lymph node negative gastric carcinoma patients using Cox proportional hazard regression model

| Variables | Risk ratio | 95% CI | P value |
|---|------------|-----------|---------|
| Age(yr) (<65 vs ≥65) | 1.354 | 0.95-1.92 | NS |
| Gender (male vs female) | 0.923 | 0.66-1.28 | NS |
| Location (upper vs distal) | 0.731 | 0.49-1.09 | NS |
| Tumor size (mm) (<50 vs ≥50) | 1.513 | 1.06-2.21 | <0.05 |
| Histologic type (differentiated vs. undifferentiated) | 0.749 | 0.55-1.02 | NS |
| Serosal invasion (negative vs positive) | 3.409 | 2.42-4.80 | <0.001 |
| Extent of lymph node dissection (<D2 vs ≥D2) | 1.188 | 0.56-2.50 | NS |
| Curability (curative vs non-curative) | 3.84 | 2.11-7.00 | <0.001 |
| Esophageal invasion (negative vs positive) | 1.007 | 0.34-2.97 | NS |

CI, confidence interval; NS, not significant.

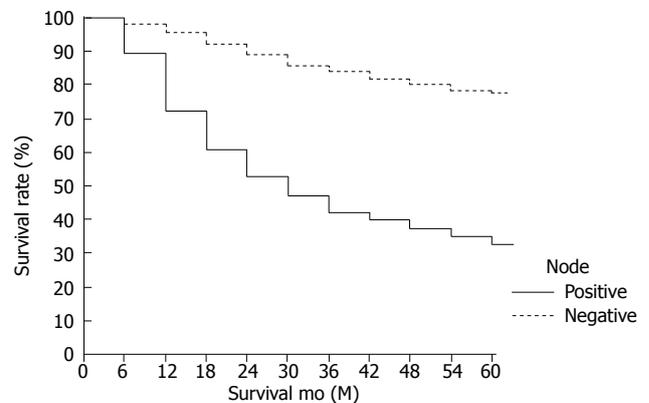


Figure 1 Survival curves of patients with lymph node-negative and node-positive gastric carcinoma (node-negative, 77.4% vs node-positive, 31.1%) ($P < 0.001$).

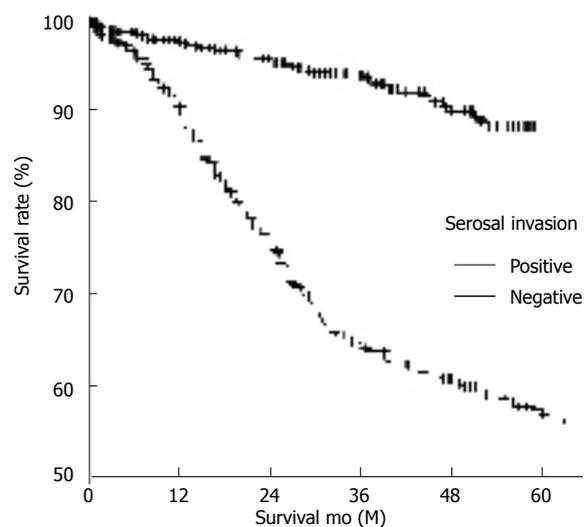


Figure 2 Survival curves of lymph node-negative gastric carcinoma according to serosal invasion (positive, 55.4% vs negative, 88.4%) ($P < 0.001$).

curability emerged as independent, statistically significant, prognostic indicators (Table 4). The survival curves according to serosal invasion, tumor size, and curability for patients with lymph node-negative gastric carcinoma are presented in Figures 2-4.

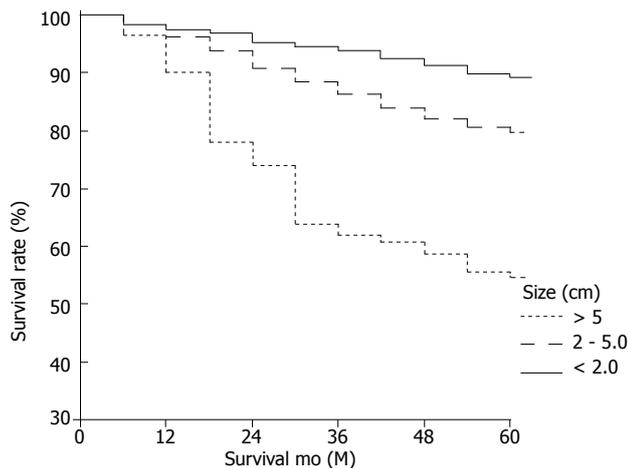


Figure 3 Survival curves of lymph node-negative gastric carcinoma according to tumor size (<math>< 2</math>cm, 88.5% vs 2-5 cm, 79.2% vs

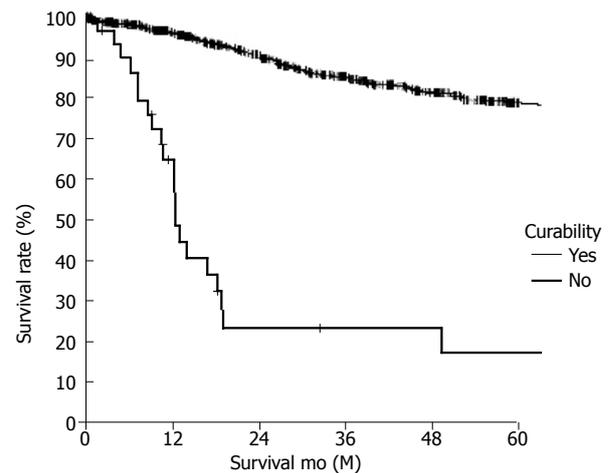


Figure 4 Survival curves of lymph node-negative gastric carcinoma according to curability (curative, 78.9% vs non-curative, 17.4%) (

DISCUSSION

In Korea, gastric carcinoma is the leading cause of death as a result of a malignant neoplasm. Although most patients with lymph node-negative gastric carcinoma have a better prognosis than lymph node-positive ones, some lymph node-negative patients have recurrence and poor survival. The identification of factors associated with poor survival in patients with lymph node-negative gastric carcinoma is important. In this study, we compared node-negative and node-positive patients in order to identify the clinicopathological characteristics of lymph node-negative gastric carcinoma. We also evaluated outcome indicators in lymph node-negative patients.

In addition to lymph node invasion, the depth of wall invasion emerged as one of the most important prognostic indicators of lymph node-negative gastric carcinoma^[12]. Adachi *et al*^[13] reported that the depth of wall invasion provided useful prognostic information in patients with gastric carcinoma. In the present study, serosal invasion also emerged as a statistically significant independent prognostic indicator using the Cox proportional hazard regression model.

Whether age is a prognostic indicator for lymph node-negative gastric carcinoma is controversial^[5,14]. Moriguchi *et al*^[15] reported that age at operation was a significant prognostic indicator in patients with early gastric carcinoma. Similarly, Adachi *et al*^[13] reported that patient's age was the second most important prognostic indicator in patients with lymph node-negative gastric carcinoma. Furthermore, some investigators have reported that survival rates were lower in elderly patients with gastric carcinoma^[16,17]. Contrary to the aforementioned reports, our study of patients with lymph node-negative gastric carcinoma revealed that age did not affect the survival rate.

Whether tumor size independently correlates with the prognosis for gastric carcinoma is also controversial. In the present study, there was a significant difference in tumor size between lymph node-negative and lymph node-positive patients (2.9cm vs 5.0cm), and node-negative patients with large tumors had poor survival. Some investigators have stressed that tumor size is an

independent prognostic indicator for gastric carcinoma, whereas others believe that tumor size does not independently influence survival. Adachi *et al*^[13] reported that tumor size served as a simple prognostic indicator for gastric carcinoma. By contrast, Yokota *et al*^[18] reported that the presence of lymph node metastasis, depth of invasion, and tumor location were more important prognostic indicators than tumor size. Maruyama^[2] reported findings that supported the conclusions of Yokota *et al*.

Surgical resection is the only potentially curative modality for localized gastric carcinoma. Adachi *et al*^[13] stated that it was reasonable to conclude that the extent of lymph node dissection did not influence the survival of patients without lymph node metastasis. They stressed that the macroscopic diagnosis of lymph node involvement was unreliable and recommended D2 lymph node dissection for curative treatment of node-negative gastric carcinoma. Harrison *et al*^[19] also recommended extended lymph node dissection for the improvement of survival of patients with lymph node-negative gastric carcinoma. We concurred with the aforementioned recommendations, and performed dissection above the D2 lymph node in most patients with lymph node-negative gastric carcinoma. In accordance with most reports, curative resection offered the only chance of long-term survival. In our series, this approach allowed us to achieve a high rate of curative resection (97.0%) and favorable outcomes in the lymph node-negative patients.

In this study, 5-year survival rates were different between patients with lymph node-negative and lymph node-positive gastric carcinoma. The 5-year survival rate was significantly lower for lymph node-positive patients than for lymph node-negative ones (35.8% vs 79.2%). Bruno *et al*^[8] and Harrison *et al*^[19] reported overall survival rates of 72% and 79%, respectively, for patients with lymph node-negative gastric carcinoma. Many factors influence the 5-year survival rate. Yokota *et al*^[20] reported that tumor size, vascular microinvasion, and the cancer-stromal relationship were the most reliable predictors of 5-year survival for patients with lymph node-negative gastric carcinoma, while Adachi *et al*^[21] reported that depth of wall invasion and patient's age were the most important prognostic

indicators. Bruno *et al*^[8] found that serosal invasion, residual disease, and poor differentiation were independent prognostic indicators of gastric carcinoma. Our univariate analysis revealed that age, tumor size, depth of invasion, tumor location, operative type, and tumor stage at initial diagnosis were prognostic indicators of lymph node-negative gastric carcinoma. In addition, in the present study, the Cox proportional hazard regression model revealed that tumor size, presence of serosal invasion, and curability were prognostic indicators of lymph node-negative gastric carcinoma.

In conclusion, we found that lymph node-negative gastric carcinoma is associated with a favorable outcome. We also found that tumor size, serosal invasion, and curative resection are the most reliable predictors of long-term survival for patients with lymph node-negative gastric carcinoma.

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P120ctn overexpression enhances β -catenin-E-cadherin binding and down regulates expression of survivin and cyclin D1 in BEL-7404 hepatoma cells

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Abstract

AIM: To understand the role of P120ctn in E-cadherin-mediated cell-cell adhesion and signaling as well as in hepatoma cell biological function.

METHODS: We stably overexpressed p120ctn isoform 3A in BEL-7404 human hepatoma cells and studied the effect of p120ctn on β -catenin and E-cadherin binding as well as p120ctn and β -catenin subcellular localization using immunoprecipitation, Western blotting and confocal microscopy. We also investigated the inhibitory effect of p120ctn transfection on the expression of apoptotic protein survivin and cell cycle regulator cyclin D1 in the cells.

RESULTS: Western blotting indicated that p120ctn expression increased after cells were transfected with p120ctn isoform 3A. The protein was located mainly at membrane under immunofluorescent microscope. β -catenin nuclear expression was reduced after overexpression of p120ctn isoform 3A. The p120ctn-E-cadherin binding increased after transfection of p120ctn isoform 3A. Furthermore, overexpression of p120ctn down regulated the expression of apoptotic protein survivin and cell cycle regulator cyclin D1. These effects led to reduction of cell proliferation.

CONCLUSION: Our results indicate that p120ctn plays an important role in regulating the formation of E-cadherin and β -catenin complex, cell apoptosis, cell cycle and cancer cell biological function.

INTRODUCTION

P120ctn is a catenin family member^[1,2]. Studies indicated that p120ctn plays an important role in E-cadherin-mediated epithelial cell adhesion and cell signaling^[3-6]. Recent studies demonstrated that p120ctn plays a critical role in certain cancerous diseases^[7-13]. Nuclear translocation of β -catenin is correlated with tumor progression and has been defined as an oncogene^[14]. P120ctn acts as a modulator in E-cadherin-mediated cell-cell adhesion and signaling including β -catenin turnover^[11,15]. However, the exact role of p120ctn in cell biological function remains unclear. Tyrosine phosphorylation of p120ctn enhances its nuclear translocation and displays hepatoma cell malignant features^[16,17]. In order to further understand the role of p120ctn in E-cadherin-mediated cell biological function and its mechanism, we studied the effects of p120ctn overexpression on β -catenin subcellular localization, regulation of cell cycle and apoptotic proteins as well as cell proliferation in hepatoma cells.

MATERIALS AND METHODS

Cell culture

BEL-7404 human hepatoma cell line was purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences in Shanghai. Cells were maintained in RPMI 1640 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 100 mL/L of fetal bovine serum, 10000 U/L of penicillin and 10000 μ g/L of streptomycin, in humidified atmosphere containing 50 mL/L of CO₂ at 37 °C.

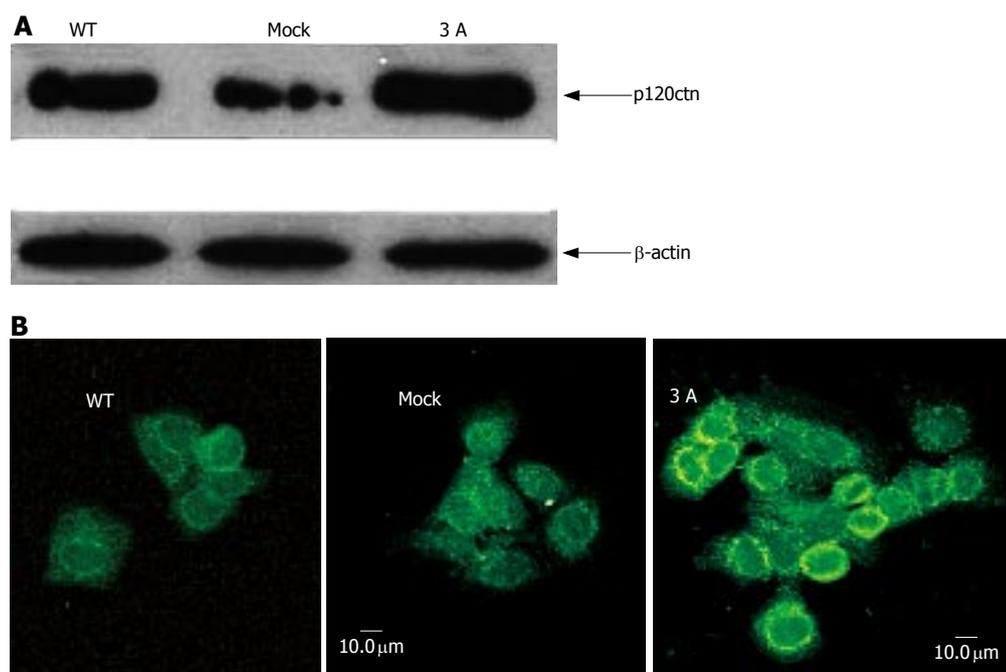


Figure 1 Western blotting (A) and confocal microscopy (B) showing increased expression of p120ctn and its subcellular localization after cells transfected with p120ctn isoform 3A.

DNA transfection

P120ctn isoform 3A plasmid and empty vector were a generous gift from Dr. Albert B. Reynolds in the department of Cancer Cell Biology, Vanderbilt University, USA. Plasmid DNA was propagated using a conventional protocol. Lipofectamine transfection reagent (Gibco BRL) was used following the manufacturer's instructions. G418 (Gibco BRL) was used as a selection antibiotic.

Immunoprecipitation and Western blot

Cells were lysed in lysis buffer containing 40 mmol/L Na_2PO_4 (pH 7.2), 250 mmol/L NaCl, 50 mmol/L NaF, 5 mmol/L EDTA, 10 mL/L Triton X-100, 10 mL/L deoxycholate (Sigma-Aldrich, St. Louis, MO, USA) for 20 min on ice. Cellular debris and nuclei were removed by centrifugation at 13 000 r/min for 15 min at 4 °C. Protein concentration was determined using Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA), 1 000 μg of total protein from each sample was immunoprecipitated with 10 μg of anti-E-cadherin monoclonal antibody (BD Pharmingen, San Diego, CA, USA), and 50 μL of protein G agarose (Gibco BRL) was added and samples were mixed by rotation at 4 °C for 1 h. The beads were pelleted and washed four times, then 50 μL of 2×SDS sample buffer was added and boiled for 5 min, and 30 μL of supernatant was loaded to SDS-PAGE gel for protein resolving. Proteins were then transferred to PVDF membrane (Bio-Rad) and incubated with anti-p120ctn (BD Pharmingen), anti-β-catenin (BD Pharmingen) or anti-survivin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-cyclin D1 (Santa Cruz) for Western blotting at 4 °C overnight. Membranes were incubated with HRP-conjugated secondary antibody at room temperature for 1 h, and illuminated by ECL solution (Amersham Biosciences, Piscataway, NJ, USA). Protein bands were visualized using Kodak X-ray film and processed by Kodak film processor (Kodak, Rochester, NY, USA).

Confocal microscopy

Cells were grown on coverslips coated with poly lysine or laminin. The cover slips were rinsed with PBS, then fixed with 12 g/L of formaldehyde at room temperature for 15 min, followed by blocking with 30 mL/L BSA at room temperature for 30 min, the cover slips then were incubated with primary antibodies for 1 h at room temperature and were washed with TBS-T and incubated with FITC-conjugated secondary antibody for 1 h at room temperature. The cover slips were mounted on slides and examined under a Leica laser confocal microscope.

Cell proliferation assay

A total of 200 000 of different types of cells were seeded into 60 mm dishes and grown for 3 d. Cells were trypsinized and trypan blue exclusion assay was used in cell counting. Experiment was performed in triplicate.

RESULTS

P120ctn expression after transfection

BEL-7404 cells were stably transfected with p120ctn isoform 3A, and the transfection efficiency was determined by Western blotting using mouse anti-p120ctn antibody. The result showed that the expression of p120ctn increased after transfection (Figure 1A). Confocal microscopy result indicated that membranous and cytoplasmic expression of the protein increased mainly at cell-cell contact region (Figure 1B).

Effect of transfection on the binding of p120ctn to E-cadherin

In order to understand the effect of p120ctn overexpression on the binding of p120ctn to E-cadherin, we immunoprecipitated E-cadherin and immunoblotted it with anti-p120ctn. The result showed that the level of p120ctn in E-cadherin-p120ctn complex was increased (Figure 2).

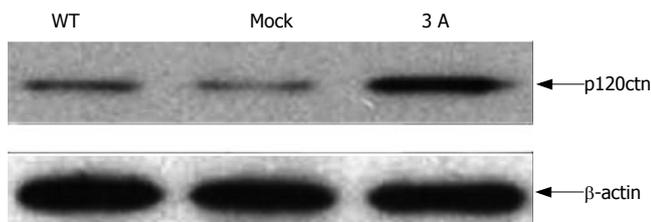


Figure 2 Binding of p120ctn to E-cadherin after cells transfected with p120ctn isoform 3A.

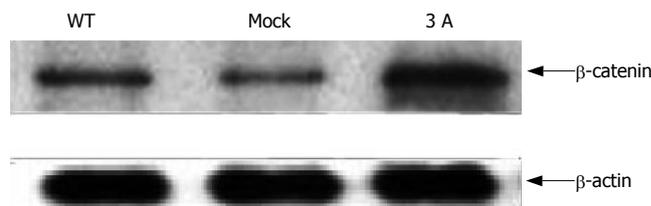


Figure 4 Binding of β -catenin to E-cadherin after cells transfected with p120ctn isoform 3A.

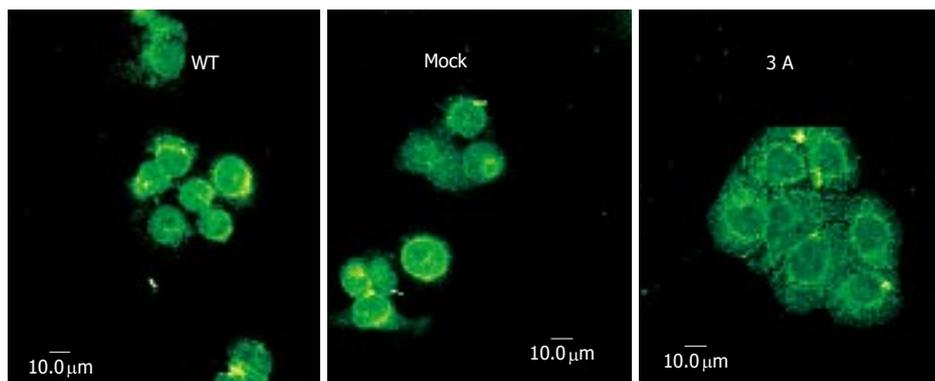


Figure 3 Changes of β -catenin subcellular localization after cells transfected with p120ctn isoform 3A.

Subcellular relocation of β -catenin after overexpression of p120ctn

In order to understand the relationship between p120ctn and β -catenin in E-cadherin-mediated cell adhesion and signaling, we examined the alteration of β -catenin subcellular localization after overexpression of p120ctn isoform 3A under confocal microscope. Figure 3 shows the changes of β -catenin expression pattern, namely the obvious expression of membranous protein and the reduction of nuclear expression.

Effect of p120ctn overexpression on the binding of β -catenin to E-cadherin

Since overexpression of p120ctn reduced the nuclear but increased membranous β -catenin expression, we immunoprecipitated E-cadherin and immunoblotted it with anti- β -catenin. The result showed that the expression level of β -catenin in E-cadherin and β -catenin complex increased after p120ctn isoform 3A transfection (Figure 4).

P120ctn transfection down regulated survivin expression

Survivin could be highly expressed in cancer but undetectable in nonproliferating normal adult tissues^[18-20]. Our Western blot result showed that overexpression of p120ctn reduced survivin expression, in some extent, in the cells (Figure 5A).

P120ctn expression down regulated cyclin D1 expression

We performed Western blotting to detect cyclin D1 expression after cells were transfected with p120ctn isoform 3A. The result indicated that cyclin D1 was down regulated after transfection (Figure 5B).

Effect of p120ctn transfection on cell proliferation

Our above results showed that overexpression of

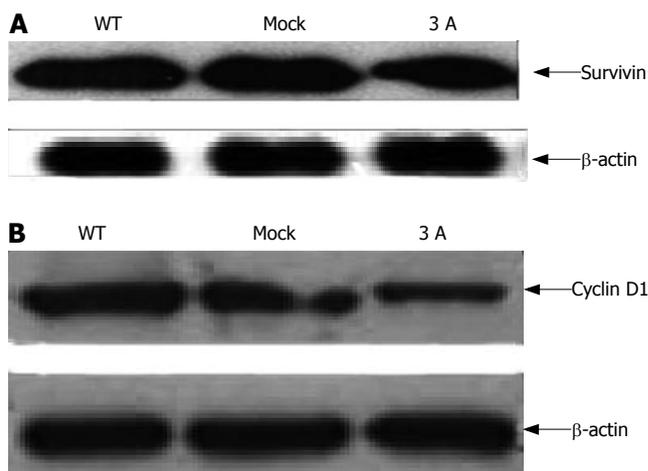


Figure 5 Down regulation of survivin (A) and cyclin D1 (B) expression after transfection of p120ctn.

p120ctn could reduce β -catenin nuclear expression, enhance binding of p120ctn and β -catenin to E-cadherin. Moreover, overexpression of p120ctn isoform 3A could down regulate survivin and cyclin D1 expression in cells. Thus, the effect of p120ctn overexpression on the proliferation of BEL-7404 hepatoma cells was examined. Using trypan blue exclusion cell counting method, we found that transfection of p120ctn decreased cell proliferation (Figure 6).

DISCUSSION

P120ctn binds to the cytoplasmic tail of E-cadherin in epithelial cells^[1,4]. Studies indicated that perturbing p120ctn-E-cadherin binding can lead to nuclear translocation and affect cell biological behavior^[21-23],

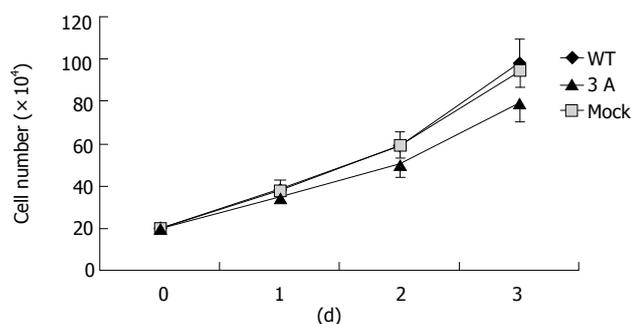


Figure 6 Effect of p120ctn transfection on cell proliferation.

suggesting that p120ctn acts as an oncogenic protein like β -catenin. On the other hand, alterations in E-cadherin and its cytoplasmic regulators, catenins, have been implicated as central to this process, and p120-catenin is frequently altered and/or lost in tumors of the colon, bladder, stomach, breast, prostate, lung, and pancreas^[5,11]. Moreover, loss of p120ctn appears to be an early event in tumor progression, possibly preceding loss of E-cadherin, suggesting that p120 plays a role as a “dual modulator” in different cell types^[5,7].

In our study, overexpression of p120ctn isoform 3A could enhance its binding to E-cadherin in BEL-7404 human hepatoma cell line, the expression was mainly found at membrane, and cytoplasmic expression could be seen as a minor event. Our results are consistent with a previous report^[17], indicating that p120ctn binds to E-cadherin, forming an E-cadherin-p120ctn adhesion complex.

In order to better understand the relationship between p120ctn and β -catenin in E-cadherin-mediated cell-cell adhesion and signaling in liver cancer cells, we examined β -catenin subcellular localization and its binding to E-cadherin after transfection of p120ctn isoform 3A. Our results showed that β -catenin translocation could be seen after transfection of p120ctn. β -catenin became nuclear “exporting”, namely its nuclear expression was reduced while membranous and cytoplasmic expression was increased. Transfection of p120ctn also increased β -catenin binding to E-cadherin, demonstrating that p120ctn can recruit β -catenin from nucleus to cytoplasm and strengthen the formation of E-cadherin-catenin complex. Since β -catenin is an oncoprotein, it binds to the TCF/LEF transcription factor and triggers cell cycle progression, thus influencing cell biological function. Our results are consistent with a previous report^[22]. We were unable to detect other pathways of the turnover of nuclei-exported β -catenin, such as APC or ubiquitously degradation pathway due to certain study limitations.

It was reported that β -catenin and p120ctn play an important role in cell proliferation, and TCF/ β -catenin signaling participates in regulation of survivin transcription in colon cancer^[23,24]. Monoclonal anti-Wnt-2 antibody induces melanoma cell apoptosis by inactivating survivin^[25]. Pizem *et al*^[26] reported that the expression of survivin is associated with aberrant activation of the WNT (wingless) pathway in medulloblastoma patients. Our results indicated that overexpression of p120ctn could down regulate survivin expression, which could be

explained by the fact that survivin is reduced due to the recruitment of β -catenin to E-cadherin- β -catenin complex and the free portion of β -catenin could be degraded by APC and ubiquitization after p120ctn overexpression, leading to decrease of nuclear β -catenin and transcription factor inactivation.

β -catenin plays an important role in cell biological function^[27,28]. It was reported that cyclin D1 transcription involves Wnt signaling in gastric cancer cell line^[29]. In skeletal myocytes, beta-catenin overexpression increases proliferation and cyclin D1 expression while decreases apoptosis and induces hypertrophy^[30]. It has been shown that in HeLa and squamous cells, differentiation-inducing factor-1 inhibits tumor cell proliferation and reduces the expression of cyclin D1 mRNA and the amount of beta-catenin^[31], suggesting that there is a close correlation between β -catenin and cyclin D1 in tumor cell biology. In our study, transfection of p120ctn isoform 3A could reduce cyclin D1 expression, resulting in the decrease of hepatoma cell proliferation.

In conclusion, overexpression of p120ctn in hepatoma cells can recruit β -catenin from nucleus to membrane and cytoplasm, enhanced E-cadherin-catenin adhesion complex, down regulated apoptosis related protein survivin and cell cycle related protein cyclin D1 expression, and finally, inhibited cell proliferation.

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COLORECTAL CANCER

Pedigree and genetic analysis of a novel mutation carrier patient suffering from hereditary nonpolyposis colorectal cancer

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hMSH2 gene might be responsible for the development of colon cancers. In family members where the exon 7 mutation is not coupled with this missense mutation, colon cancer appears after the age of 40. The association of these two mutations seems to decrease the age of manifestation of the disease into the early thirties.

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Key words: Hereditary nonpolyposis colon cancer; Bethesda criteria; Mutation; hMLH1; hMSH2

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Abstract

AIM: To screen a suspected Hungarian HNPCC family to find specific mutations and to evaluate their effect on the presentation of the disease.

METHODS: The family was identified by applying the Amsterdam and Bethesda Criteria. Immunohistochemistry was performed, and DNA samples isolated from tumor tissue were evaluated for microsatellite instability. The identification of possible mutations was carried out by sequencing the hMLH1 and hMSH2 genes.

RESULTS: Two different mutations were observed in the index patient and in his family members. The first mutation was located in exon 7, codon 422 of hMSH2, and caused a change from Glu to STOP codon. No other report of such a mutation has been published, as far as we could find in the international databases. The second mutation was found in exon 3 codon 127 of the hMSH2 gene, resulting in Asp→Ser substitution. The second mutation was already published, as a non-pathogenic allelic variation.

CONCLUSION: The pedigree analysis suggested that the newly detected nonsense mutation in exon 7 of the

INTRODUCTION

According to published data, about 15-20 % of colorectal carcinomas (CRC) follow a familial pattern. Familial adenomatous polyposis coli syndrome (FAP) and its subtypes are responsible for only about 1 % of all CRCs, while hereditary nonpolyposis colon cancer (HNPCC) accounts for approximately 3-8 % of cases^[1-3]. The characteristic presentation of HNPCC is frequently right-sided localization, the presence of synchronous and metachronous CRCs, and its association with other HNPCC-related extracolonic tumors, including gastric, endometrial, and urinary and biliary tract cancers in afflicted families^[2,3]. Compared to sporadic colorectal carcinomas, HNPCC has an earlier age of onset, Crohn's disease-like lymphocytic infiltration in tumor tissues, increased mucin production, and a lower degree of histological differentiation^[3-5]. The classic adenoma-carcinoma sequence can be observed in HNPCC patients as well, but the conversion of polyps to malignant proliferation is accelerated, taking only 1-3 years, as opposed to sporadic adenomas, where this can take as long as 10-15 years^[1]. The number of polyps in the colon is not so high as in cases of FAP. The genetic background of HNPCC has been identified in the germline defect of DNA mismatch repair genes (MMR). The syndrome is in-

herited in an autosomal dominant fashion, with an estimated penetrance of 80%^[1-3,6]. At present, seven MMR genes are known (hMLH1, hMLH3, hMSH2, hMSH3, hMSH6, hPMS1, hPMS2); however, the influence of a specific mutation on the pathogenesis of the disease shows great diversity^[7-10]. In about 90-95% of the cases, germline mutations of the hMLH1 or hMSH2 genes can be demonstrated, while only a low percentage is caused by the mutations of hMSH6, hPMS1 and hPMS2^[1]. The detection of afflicted families must be initiated by taking a thorough family history, embracing at least three generations, applying the widely accepted Amsterdam and Bethesda Criteria^[6]. If the diagnosis of HNPCC is further supported by the immunohistochemistry of the MMR proteins, the microsatellite status must be determined. The hallmark of mismatch repair deficiency is microsatellite instability (MSI), variability of the lengths of short repetitive DNA stretches scattered in the genome (microsatellites) compared to normal tissue DNA^[11-14]. Finally, sequencing of the MMR genes can accurately identify mutations and makes screening of other family members possible^[6]. It is emphasized in the literature that only around 40% of the families identified by the Amsterdam Criteria will actually present detectable mutations. This fact influences both therapeutic plans and the required long-term follow-up^[3,13,15,16].

In this study, our goal was to perform the genetic analysis of a young male patient and the members of his family who had been selected by the Amsterdam Criteria, and to identify the genetic alterations in the MMR genes hMLH1 and hMSH2. We also attempted to establish a correlation between the occurrence of mutations and disease pattern.

MATERIALS AND METHODS

Patient and surgery

A 32-year-old male patient (index person) with recurrent hematochesia was received at an outpatient clinic in North-Eastern Hungary. Colonoscopy was performed and a friable, bleeding lesion was found at the ileal flexure of the large bowel with circular narrowing of the lumen. The histopathological report of the biopsy showed a mucinous adenocarcinoma infiltrating the muscularis propria, pT2, Grade 2. Ultrasound and computer tomography ruled out evidence of liver metastases, enlarged lymph nodes in the mesocolon or spread to neighboring organs. Since the disease was localized, a left hemicolectomy was performed with an end-to-end transverso-rectostomy. The family and the index person were evaluated using the Amsterdam and Bethesda Criteria^[2]. Based on the family history, the possible diagnosis of HNPCC was suspected and, following discussions about the disease, the patient and his family agreed to further genetic evaluation.

Immunohistochemistry

Routine 5 μ m, paraffin-embedded tissue sections were dewaxed, rehydrated, and treated in a microwave oven in 10 mmol/L citrate buffer (pH 6.4) for 20 min, in order to retrieve antigenicity. Unspecific protein binding was blocked with 1% bovine serum albumin containing PBS for 30 min at 37 °C, then slides were incubated overnight with the pri-

mary antibodies (clone 2 MSH01, mouse monoclonal anti-MSH2, 1:100 and clone M074581 mouse monoclonal anti-MLH1, Labvision Corp., Fremont, CA, USA), respectively. Primary antibodies were detected by a biotin-streptavidin detection kit (LSAB, Dako, Carpinteria, CA, USA) using VIP chromogen. Negative controls were stained with the omission of the primary antibodies.

DNA extraction

DNA was extracted from a paraffin-embedded cancerous tissue sample of the index patient following deparaffinization and proteinase K digestion, according to the protocol of the High Pure PCR Template Purification kit (Roche Diagnostics GmbH, Mannheim, Germany). DNA samples of the patient and family members were also isolated from whole blood using the Wizard DNA Purification System (Promega Corporation, Madison WI, USA) according to Promega's Technical Manual (TM050).

Microsatellite analysis

DNA samples isolated from the tumor tissue were tested for microsatellite instability. Two mononucleotide markers (BAT25 and BAT26) were evaluated using the technique described by Dietmaier *et al*^[17]. A multiplex PCR was performed using sequence specific hybridization probes (HyProbes) labeled with LightCycler dyes, LCRed640 and LCRed705. Amplification of microsatellites by LightCycler (Roche Diagnostics GmbH, Germany) was followed by melting point analysis to display alterations in the length of repetitive sequences.

Heteroduplex and single strand conformation polymorphism (SSCP) analysis

All exons of hMLH1 and hMSH2 genes were analyzed in the blood sample of the index patient. Primers and PCR conditions were used as previously described^[18]. Primers are available upon request. After denaturation or heteroduplex formation, the PCR products were loaded to electrophoresis on MDE gel (Cambrex Bio Science Rockland Inc., Rockland, MA, USA) according to manufacturer's instructions and visualized by silver staining.

Sequencing of hMSH2 and hMLH1 genes

PCR products showing altered migration patterns on MDE gel were purified and sequenced in both directions. Sequencing reactions were performed using a BigDye thermocycler sequencing kit v.3.1 (Applied Biosystems, Foster City, CA, USA). The semi-automated fluorescence analysis was performed with an ABI-PRISM 310 Genetic Analyzer (Perkin Elmer, Boston, MA, USA).

RESULTS

After a comprehensive evaluation of three generations, the index person's family was found to fulfill several points of the Amsterdam and Bethesda Criteria. In the maternal line of the pedigree, seven persons carry the exon 7 mutation. In this line, four cases of colorectal cancer and one case of breast cancer were recognized spanning three generations among first degree relatives. Analyzing the history of the paternal line, pulmonary carcinoma and gastric carcinoma

Table 1 Details of the pedigree analysis

| Classification | Degree of relationship | 7 exon mutation | 3 exon mutation | Type of the tumor, age at tumor detection | Age at investigation |
|----------------|--|-----------------|-----------------|---|----------------------|
| I/1 | Sister of the mother of the index person's mother | | | Breast cancer, 61 yr | Died |
| I/2 | Mother of the index person's mother | | | | 76 yr |
| I/3 | Brother of the father of the index person's mother | + | | CRC, 56 yr | 68 yr |
| I/4 | Sister of the father of the index person's mother | + | | | 61 yr |
| I/5 | Husband of the person No I/4 | | + | | 64 yr |
| I/6 | Mother of the index person's father | | | Gastric cancer, 55 yr | Died |
| I/7 | Father of the index person's father | | | Lung cancer, 75 yr | Died |
| II/1 | Index person's mother | + | | CRC, 43 yr | 54 yr |
| II/2 | Index person's father | | + | | 58 yr |
| II/3 | Sister of index person's father | | + | | 48 yr |
| II/4 | The middle son of person No I/4 | + | + | CRC, 34 yr | 36 yr |
| II/5 | The youngest son of person No I/4 | + | | | 32 yr |
| II/6 | The oldest son of person No I/4 | | | | 39 yr |
| III/1 | Index person | + | + | CRC, 31 yr | 33 yr |
| III/2 | Index person's younger brother | + | + | | 28 yr |
| IV/1 | Older son of the index person | | | | 3 yr |
| IV/2 | Younger son of the index person | | | | 1 yr |

CRC:colorectal carcinoma.

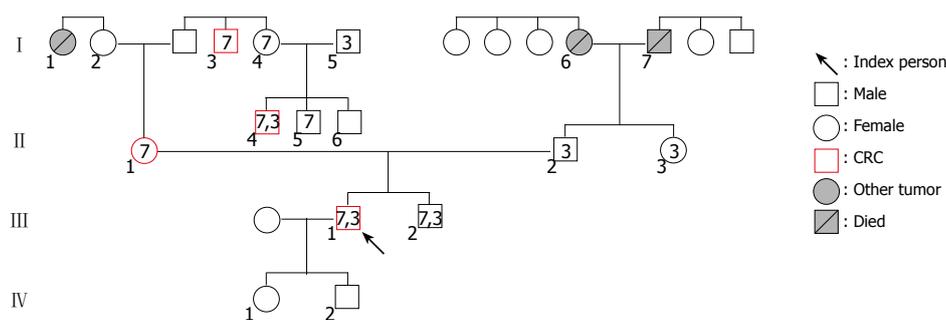


Figure 1 Pedigree of the index person.

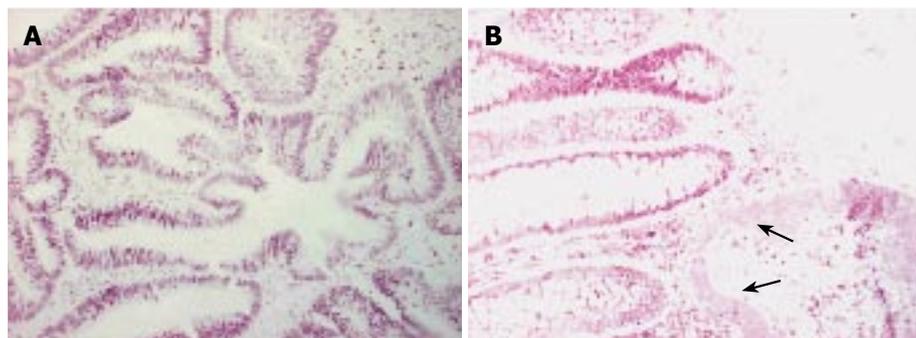


Figure 2 Immunostaining for hMLH1 and hMSH2. Nuclei of tumor and normal colon mucosa cells show reactivity for hMLH1 (left), whereas hMSH2 staining is missing from the tumor cells (right, arrows). LSAB, original magnification, x200.

(one case each) were detected (Figure 1 and Table 1). We found two generations (I./5 and II./2) in the maternal line, where exon 3 mutations join from two different families. In both cases of mutation associations, the tumor manifestation could be observed at a younger age than in the other family members, who carried the exon 7 mutation only. Numbers in icons mean the location of hMSH2 exon mutations. Numbers below icons identify the investigated family members. The younger brother of the index person (III./2.) was 28 years old at the time of the investigation and was disease-free.

Immunohistochemistry of the index person's tumor tissue showed a complete loss of hMSH2 reactivity. The hMLH1 staining was retained in the nuclei of the tumor cells (Figure 2). Furthermore, this tumor tissue was also proved to be MSI-High, as was demonstrated by melting point analysis of both mononucleotide markers, BAT25 and BAT 26 (Figure 3).

Sequencing of all the exons of hMSH2 and hMLH1 genes from the same tumor tissue showed two mutations of the hMSH2 gene. We were not able to demonstrate any alterations in the hMLH1 gene. The first mutation was a

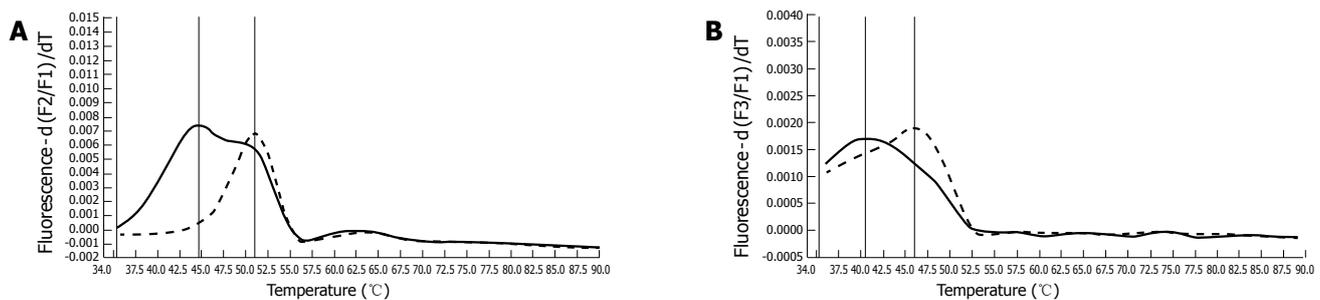


Figure 3 LightCycler T_m estimation of Bat26 (A) and Bat25 (B) microsatellite markers. Dotted lines represent MSS control. T_m is 51°C for Bat26 and 46°C for Bat25. The solid lines show decreased T_m values corresponding to the MSI-H status of tumor tissue. T_m is 44.6°C for Bat26 and 40.5°C for Bat25 respectively.

Table 2 Type and location of detected mutations

| Gene | Exon | Codon | Mutation | Type |
|-------|------|-------|---------------------|----------|
| hMSH2 | 7 | 422 | gaa→taa Glu→STOP | Nonsense |
| hMSH2 | 3 | 127 | aat→agt Asn→Ser | Missense |

nonsense mutation, located in exon 7, codon 422 causing a glutamine→STOP codon change (Table 2). The second one was a missense mutation, located in exon 3, codon 127 resulting in substitution of Asp for Ser.

Targeted sequencing of these two identified mutations was performed on DNA samples isolated from the peripheral blood leukocytes of fourteen other family members. On the paternal side, the missense mutation of exon 3 was identified in the index patient's younger brother and his father, as well as in the sister of the father. Interestingly, the exon 3 mutation was also found in two members of the maternal line. The nonsense mutation of exon 7 was found in seven members of the maternal side and four of these subjects have been diagnosed as CRC (Table 1).

DISCUSSION

A characteristic feature of HNPCC is a tendency to develop metachronous tumors in affected individuals. In a 15-year follow-up study, Jarvinen and coworkers demonstrated convincingly that recurrence of CRC can be eliminated effectively by regular interval colonoscopies and polypectomies. Out of the 133 individuals who underwent regular interval follow-up examinations, 18% developed colon cancer, but no patients died. Whereas, among the 119 patients who deferred colonoscopy at three-year intervals, 41% were diagnosed as CRC and 9 deaths occurred^[19]. Since the correct identification of HNPCC families is still an unsolved issue, considerable effort is dedicated to the task of uncovering further details of the disease. The most widely applied initial screening tools are the Amsterdam and Bethesda Criteria^[2,3,6]. Unfortunately, neither of them is accurate enough to identify all HNPCC families with complete certainty. The most reliable, currently available technique is the demonstration of mutations in the MMR genes of suspected index persons, followed by a comprehensive search for the specific mutation in their family members. A promising way to reach an effective screening

tool is to find and describe the nature and frequency of population specific mutations and to test these first in patients from a particular geographic location. Currently, more than 300 different MMR gene mutations have been published from different parts of the world. Some of these are recurrent, and have been described as founder mutations in particular populations^[20-24].

Our group, applying the previously suggested protocol, initiated an investigation in Hungary. A 31-year-old male patient was selected by the Bethesda criteria and genetic testing identified him as having HNPCC. A double mutation of the hMSH2 gene was found and subsequently compared with the available international databases (The Human Gene Mutation Database, Cardiff, International Society for Gastrointestinal Hereditary Tumors). The nonsense mutation of exon 7 has not been published before; therefore, it may potentially be characteristic in Hungarian families. The genetic error clearly appears to be pathogenic, since all the family members with CRC carry this mutation. The newly identified mutation causes development of a STOP codon, leading to a non-functional, truncated protein, which could not be detected by immunohistochemistry. The human MSH2 protein interacts with MSH6 forming the complex called hMut α and with hMSH3 protein forming the complex named hMut β . Each subunit of these heterodimers is formed by five flexible domains which are defined as I. mismatch binding, II. connector, III. core, IV. DNA clamp, and V. ATPase domains. The mismatch binding and DNA clamp domains act in binding to mismatched DNA whereas the core and connector domains constitute the backbone of each subunit. The latter two transmit allosteric information of bound DNA co-factor to the ATPase domain. Our detected mutation at codon 422 causing Glutamine→STOP change results in the loss of more than half of the expressed MSH2 protein (truncated by 513 aminoacids from 934), involving core, DNA clamp and ATPase domains. This nonfunctional truncated protein cannot take a part in forming normal heterodimers and disintegrated quite soon after expression. To the best of the authors' knowledge there was only one publication in Hungary of a novel hMSH2 germline mutation causing truncated protein expression. Czako *et al.* investigated a 62 year old female patient who suffered from colon cancer at the age of 46, rectum cancer at the age of 60, endometrial cancer at the age of 56 years, basosquamous and squamous cell cancer of the face at the ages of 53, 54, 62, and 58 years respectively. This patient's family fulfilled

the Amsterdam criteria. The DNA sequencing analysis revealed a mutation as a single nucleotide change at codon 2292 in exon 14 of the hMSH2 gene. This guanine to adenine change altered the 764 amino acid, the tryptophan to STOP codon. Thus the hMSH2 protein was truncated by 171 aminoacids^[25].

The missense mutation of exon 3 has already been described by several authors as a single amino acid change, and represents a non-pathogenic polymorphism of the gene. Samowitz *et al* published data showing 3 cases of this polymorphism in 1066 screened subjects. One of the 3 affected carriers was also found to have another missense mutation (exon 6, codon 328, Ala→Pro change, of hMSH2 gene), and in another carrier it was coupled with a pathogenic frameshift mutation of the hMLH1 gene (exon 16, codon 626).^[26] De la Chapelle *et al* published another interesting double mutation, where this polymorphism was associated with an MSH2, exon 6, codon 333 mutation (International Society for Gastrointestinal Hereditary Tumors). The allele frequency of this polymorphism, according to the database of The National Institute of Environmental Health Sciences Genome Project, is 0.02, which would lead to as much as a 4% prevalence of heterozygous carriers in the general population.

Both mutations identified in this study are single nucleotide changes of the hMSH2 gene. Large genomic deletions and/or rearrangements, on the other hand, can account for 36% of all hMSH2 mutations according to the Dutch HNPCC mutation analysis study.^[27] Unlike the hMSH2 mutations, the majority of hMLH1 mutations are single nucleotide changes, but interestingly, a 3.5 kb deletion encompassing exon 16 of hMLH1 has been observed in Finland, as a founder mutation^[23].

The presence of the exon 7 mutation alone resulted in a tumor manifestation at an age of 43 and 56 years (Table 1). When the missense and nonsense mutations were inherited together, the age of manifestation shifted to a younger age of 31 and 34 years.

It is well known that the penetrance of the MMR genes is less than 100%, which might explain why two of the older family members carrying the pathogenic mutation have not acquired the disease yet. Young age may answer the same question in the case of the younger brother of the index person, who was 28 years old at the time of the investigation (Table 1). On the other hand, all these family members must be considered "high-risk" patients, and be placed under close regular interval surveillance. To date, three family members carrying only the exon 3 mutation are disease-free. Three other members of the family, who suffered from breast, gastric and pulmonary cancers, respectively, could not be investigated because of their deaths before the initiation of the study.

Both the paternal and maternal lines of the evaluated family in this study came from the same small village in Hungary, which may explain the high prevalence of the exon 3 mutation of MSH2 in the tested individuals. It will be interesting to see whether the exon 7 mutation is truly characteristic for Hungarian families, or whether this mutation can be found in different populations elsewhere.

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VIRAL HEPATITIS

Distinct toll-like receptor expression in monocytes and T cells in chronic HCV infection

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INTRODUCTION

Hepatitis C virus (HCV) infection is the cause of chronic hepatitis in more than 4 million people in the USA^[1]. Patients with chronic HCV infection experience a various severity of liver diseases ranging from mild hepatitis to cirrhosis and hepatocellular carcinoma. Liver cirrhosis that appears decades after the initial infection often predisposes to spontaneous bacterial peritonitis and sepsis^[2]. HCV is the most frequent indication for liver transplantation, and transplant recipients suffer from recurrent HCV and infections associated with immunosuppression^[3]. HCV, a blood borne pathogen, is frequently associated with human immunodeficiency virus (HIV) infection acquired through the same route of invasion^[4]. According to clinical data, HCV is not only the cause of morbidity due to liver disease, but it is also associated with other infections. A recent study by El-Sarag *et al*^[5] suggested that infections with other viral and bacterial pathogens were more common in HCV-infected patients who otherwise had no immunocompromise compared to non-HCV infected controls. The pathogens causing infections at a higher rate in HCV patients included cytomegalovirus, cryptococcus, *Mycobacterium tuberculosis* and those causing sexually transmitted diseases^[5]. It remains to be evaluated whether recognition of pathogens and/or mounting appropriate immune responses is impaired in HCV infection.

Bacterial and viral pathogens are recognized as “danger” signals by the family of Toll-like receptors (TLRs) that alert innate immunity against the invading pathogens^[6]. The known 11 mammalian TLRs have distinct extracellular domains but share common intracellular mechanisms of cell activation that culminate in production of inflammatory cytokines. Each TLR has specific ligands, which allow the host to sense a wide diversity of pathogens. TLR1, TLR6 and possibly TLR10 form heterodimers with TLR2 to recognize fungi (*Sarcomyces cerevisiae*, *Candida albicans*, and *Aspergillus fumigatus*), parasites (*Trypanosoma cruzi*), Gram positive bacteria-derived peptidoglycan (PGN) and bacterial lipoproteins, lipoarabinomannan from *Mycobacterium tuberculosis*, *Treponema pallidum*-derived glycolipid

Abstract

AIM: Hepatitis C virus often establishes chronic infections. Recent studies suggest that viral and bacterial infections are more common in HCV-infected patients compared to controls. Pathogens are recognized by Toll-like receptors (TLRs) to shape adaptive and innate immune responses.

METHODS: In this study, to assess the ability of HCV-infected host to recognize invading pathogens, we investigated Toll-like receptor expression in innate (monocytes) and adaptive (T cells) immune cells by real-time PCR.

RESULTS: We determined that RNA levels for TLRs 2, 6, 7, 8, 9 and 10 mRNA levels were upregulated in both monocytes and T cells in HCV-infected patients compared to controls. TLR4 was only upregulated in T lymphocytes, while TLR5 was selectively increased in monocytes of HCV-infected patients. MD-2, a TLR4 co-receptor, was increased in patients' monocytes and T cells while CD14 and MyD88 were increased only in monocytes.

CONCLUSION: Our data reveal novel details on TLR expression that likely relates to innate recognition of pathogens and immune defense in HCV-infected individuals.

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Key words: Hepatitis C virus; Toll-like receptors; T cells; Monocytes

Table 1 Patient characteristics

| Parameter | Value |
|----------------------------------|--|
| Gender (male/female) | 12/2 |
| Age (yr) | 44 ± 12 |
| Duration of disease | > 10 yr |
| | Unknown |
| | 9 |
| Viral load (by QUNT BDNA, IU/ml) | 1 × 10 ⁷ ± 0.78 × 10 ⁷ |
| Viral genotype | (by 1a |
| PCR) | 5 |
| | 1b |
| | 4 |
| | 3a |
| | 3 |
| | 4a |
| | 2 |
| Plasma SGOT (ALT) levels (IU/mL) | 110 ± 86 |
| Plasma SGPT (AST) levels (IU/mL) | 102 ± 60 |

and lipoteichoic acid^[6,7]. TLR2 can also recognize certain viruses such as cytomegalovirus, measles virus and core and NS3 proteins of HCV^[8-10]. TLR3 recognizes poly(I-C) and double-stranded viral RNA, whereas TLR4 agonists include Gram-negative bacterial LPS, respiratory syncytial virus, *Cryptococcus neoformans* and the plant product Taxol^[6,7,11]. Bacterial flagellin has been identified as a TLR5 ligand, synthetic components imiquimod and resiquimod 848 and single stranded RNA stimulate TLR 7 and 8, while unmethylated CpG-containing DNA and herpes simplex virus have been identified as TLR9 agonists^[6,7,12].

The presence of TLRs was originally discovered in innate immune cells^[13]. Monocytes express all TLRs of which TLRs 1, 2, and 4 are present at high levels^[14]. Latest research, however, indicates that TLR expression is not restricted to innate immune cells and TLRs can be detected in adaptive immune cells as well as in parenchymal cells. In particular, T cells express all TLRs at low levels with the exception of TLR5, which is present abundantly^[14, 15].

In order to dissect the ability of HCV-infected host to respond to invading pathogens, we investigated Toll-like receptor expression in innate (monocytes) and adaptive (T cells) immune cells. We determined that TLR2, 7, 8, 9 and 10 were upregulated in both immune compartments in HCV-infected patients compared to controls. In addition, TLR4 was upregulated in lymphocytes and TLR6 was upregulated in monocytes only. Our data reveal new insights into immune defense of HCV-infected individuals.

MATERIALS AND METHODS

Blood donors

Healthy individuals (controls, *n* = 14) and treatment-naïve patients chronically infected with hepatitis C virus (HCV patients, *n* = 14) were enrolled in the study. All individuals were free of acute infections. The study was approved by the Committee for the Protection of Human Subjects in Research at the University of Massachusetts Medical School and informed consent was obtained. The clinical characteristics of the patients are detailed in Table 1. The blood was collected from cubital vein with anti-coagulant (heparin sodium) and processed immediately. Controls and patients were matched for age and gender where possible.

Cells

Peripheral blood mononuclear cells (PBMCs) were separated by centrifugation on Ficoll gradient. In order

Table 2 Real-time PCR primers

| Primer identification | Sequence |
|-----------------------|--|
| TLR1 | Forward 5'-GGG TCA GCT GGA CTT CAG AG-3' |
| | Reverse 5'-AAA ATC CAA ATG CAG GAA CG-3' |
| TLR2 | Forward 5'-GCC TCT CCA AGG AAG AAT CC-3' |
| | Reverse 5'-TCC TGT TGT TGG ACA GGT CA-3' |
| TLR3 | Forward 5'-GTG CCA GAA ACT TCC CAT GT-3' |
| | Reverse 5'-TCC AGC TGA ACC TGA GTT CC-3' |
| TLR4 | Forward 5'-AAG CCG AAA GGT GAT TGT TG-3' |
| | Reverse 5'-CTG AGC AGG GTC TTC TCC AC-3' |
| TLR5 | Forward 5'-TTG CAT CCA GAT GCT TTT CA-3' |
| | Reverse 5'-TTC AAC TTC CCA AAT GAA GGA-3' |
| TLR6 | Forward 5'-GAA CAT GAT TCT GCC TGG GT-3' |
| | Reverse 5'-GCT GIT CTG TGG AAT GGG TT-3' |
| TLR7 | Forward 5'-AAT GTC ACA GCC GTC CCT AC-3' |
| | Reverse 5'-GCG CAT CAA AAG CAT TTA CA-3' |
| TLR8 | Forward 5'-TGT GAT GGT GGT GTC TCA AT-3' |
| | Reverse 5'-ATG CCC CAG AGG CTA TTT CT-3' |
| TLR9 | Forward 5'-ATT CTG ACT TTG CCC ACC TG-3' |
| | Reverse 5'-GCT GAG GGA CAG GGA TAT GA-3' |
| TLR10 | Forward 5'-GGC CAG AAA CTG TGG TCA AT-3' |
| | Reverse 5'-AAA TGA CTG CAT CCA GGG AG-3' |
| MyD88 | Forward 5'-GAG CGT TTC GAT GCC TTC AT-3' |
| | Reverse 5'-CGG ATC ATC TCC TGC ACA AA-3' |
| MD2 | Forward 5'-ATT CCA AGG AGA GAT TTA AAG CAA TT-3' |
| | Reverse 5'-CAG ATC CTC GGC AAA TAA CTT CTT-3' |
| CD14 | Forward 5'-CGC TCC GAG ATG CAT GTG-3' |
| | Reverse 5'-TTG GCT GGC AGT CCT TTA GG-3' |

to separate monocytes from lymphocytes, the PBMCs were plated (10⁷/well in 2 mL of RPMI1640 media supplemented with 10 % FBS) in 6 well plates (Corning, Corning, NY) and incubated at 37 °C in 50 ml/L CO₂ atmosphere for 3 h. Monocytes were separated based on their adherence to plastic and the purity of the population, based on CD14 expression, was greater than 95% as determined by flow cytometry (data not shown). Non-adherent cells were washed and T lymphocytes were purified using T cell negative isolation kit (DynaL Biotech Inc, Lake Success, NY), as manufacturer recommended. Briefly, non-adherent cells were incubated with a cocktail of antibodies for CD14, CD16a, CD16b, CD56, HLA class II DR/DP and CD235A, followed by incubation with depletion magnetic Dynabeads. The non-T cells bound to the Dynabeads and were separated in a strong magnetic field while T cells were washed and subjected to RNA extraction.

Real-time PCR

Total cellular RNA was extracted using RNeasy kits (Qiagen, Valencia, CA), according to manufacturer's recommendations. All samples were co-processed to eliminate technical variations. Equal amounts of RNA from controls and HCV-infected patients were analyzed. Reverse transcription of 1 µg of RNA into cDNA was performed using reverse transcription System (Promega, Madison, WI). The real-time PCR primers were synthesized by IDT (Coralville, Iowa), except for 18S (Quantum RNA Classic II 18S Internal Standard, Ambion, Austin, TX). The real-time PCR was performed using the ICycler (Biorad Laboratories, Hercules, CA). The PCR primers are described in Table 2. The reaction mixture contained 12.5 µL qPCR

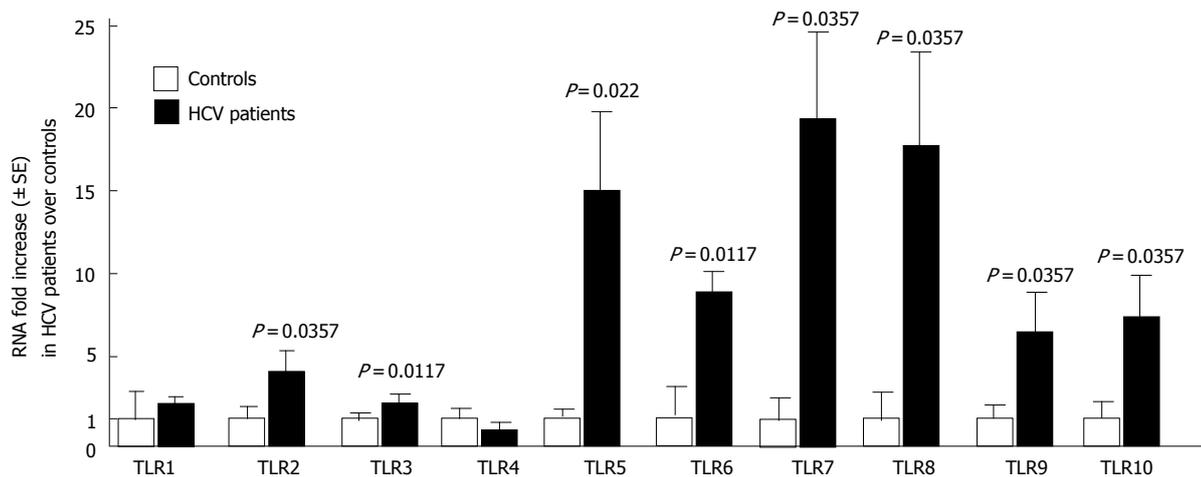


Figure 1 Higher TLRs 2, 5, 6, 7, 8, 9 and 10 RNA expression by monocytes of HCV-infected patients compared to controls. RNA was purified from adherence-isolated monocytes and real-time PCR for TLRs 1-10 and 18 S RNA (as endogenous control) was performed as described in Materials and Methods. For each individual, TLR-coding RNA expression was normalized to corresponding 18 S RNA. Results are expressed as fold increase of each TLR in HCV-infected patients compared to controls. Results are from 14 HCV-infected patients and 14 controls.

MasterMix Plus with 2 x SYBR Green (Eurogentec, Seraing, Belgium), 0.5 μ mol/L of forward and reverse primer and 1 μ L of cDNA (corresponding to 50 ng of RNA) in a total volume of 25 μ L. All amplifications and detections were carried out in a BioRad optical 96-well reaction plate with optical tape. At each cycle, accumulation of PCR products was detected by monitoring the increase in fluorescence by dsDNA-binding SYBR Green. After the PCR was performed, a dissociation/melting curve was constructed in the range of 55°C to 95°C. Data were analyzed using the Biorad ICycler software. As a first step in data analysis 18S was used for normalization of all experiments (normalizer) and it was closely compared between controls and HCV patients. For second step in data analysis we used comparative Ct method ($\Delta\Delta$ Ct method) with the following formula: Δ Ct = Ct (target, TLR) - Ct (normalizer, 18S). The comparative $\Delta\Delta$ Ct calculation involved finding the difference between Δ Ct of HCV patient and the mean value of the Δ Ct from normals for each analyzed molecule. Fold increase in the expression of specific mRNA in HCV patients compared to normal controls was calculated as $2^{-(\Delta\Delta$ Ct)}.

Statistical analysis

The non-parametric Wilcoxon test from StatView on a G4 Machintosh platform (Cupertino, CA) was employed to determine the statistical difference between controls and HCV patients.

RESULTS

HCV-infected patient selection and their characteristics

Our study population included patients with chronic HCV infection but without cirrhosis or cancer, thus, HCV infection-specific imprints on the immune system were present but cirrhosis or cancer-related non-specific immune alterations could be excluded. All patients in the study had viremia and elevated levels of liver transaminases consistent with chronic hepatitis. Patients

infected with different viral genotypes (1A, 1B, 3A and 4A) were included, as viral genotype could affect host immune response^[16]. Our patients had never been treated with anti-viral therapy, thus possible influence of therapy on TLR expression was eliminated. This is important because Ribavirin was shown to augment signaling in TLR-transfected cells^[17].

Monocytes of HCV-infected patients express higher levels of TLRs 2, 3, 5, 6, 7, 8, 9 and 10 RNA compared to controls

Chronic HCV infection is associated with increased serum levels of inflammatory cytokines^[18]. Blood monocytes and tissue macrophages are major sources of these cytokines. We recently reported increased monocyte production of TNF α in patients with chronic HCV infection^[10]. In this study, we used real-time PCR to evaluate TLR expression in monocytes of HCV-infected patients and healthy controls. Monocytes both from controls and HCV-infected patients expressed TLRs 1-10 (Figure 1), as expected based on previous studies^[14]. We found that monocytes from patients with chronic HCV infection expressed significantly higher levels of RNA coding for TLR2, TLR5, TLR6 and TLR10. Different TLRs reside at different locations in the cell. TLRs 1, 2, 4, 5, 6 and 10 are expressed mostly on the surface of the cellular membrane, while TLRs 3, 7, 8 and 9 are localized inside the cells^[6,7]. Surprisingly, we found that not only some outer cellular membrane-associated TLRs, but also the intracellularly localized TLRs, TLRs 7, 8 and 9, were upregulated in HCV patients' monocytes. The level of TLR3 mRNA was also significantly higher in HCV-infected patients compared to controls.

Ligand recognition and signaling of TLR2 and TLR4 is augmented by co-receptors^[6,7]. Thus, we further analyzed the expression of TLR co-receptors and adaptors in both controls and HCV-infected patients. We found that the mRNA level of CD14, a co-receptor for TLR2 and TLR4, was upregulated in HCV patients compared to controls (Figure 2). In addition to CD14, MD-2 is another accessory protein of the TLR4 that is necessary

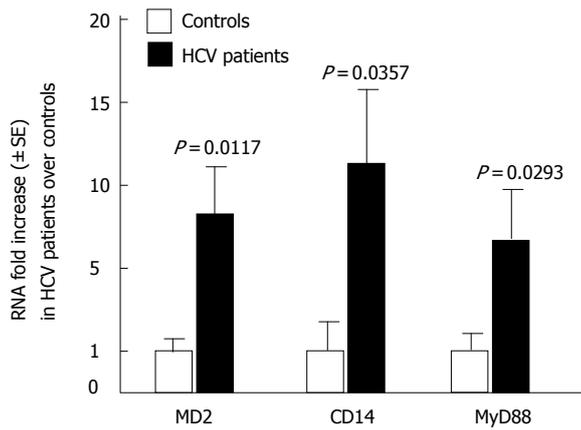


Figure 2 Monocytes of HCV-infected patients express higher levels of TLR co-receptors MD2 and CD14, and TLR adaptor MyD88 compared to controls. RNA was purified from adherence-isolated monocytes and real-time PCR for MD2, CD14, MyD88 and 18 S RNA (as endogenous control) was performed as described in Materials and Methods. For each individual protein of interest coding RNA expression was normalized to corresponding 18 S RNA. Results are expressed as fold increase of each protein of interest in HCV-infected patients compared to controls. Results are from 14 HCV-infected patients and 14 controls.

for assembling the TLR4-containing receptor complex to sense low quantities of lipopolysaccharide^[19]. We found that MD2 mRNA was expressed at significantly higher levels in monocytes of HCV infected patients compared to controls. The mRNA for MyD88, an intracellular adaptor molecule that is recruited to the intracellular domain of TLRs 2, 4, and 9 upon engagement with specific ligands, was also higher in patients compared to controls. These results suggest that increased RNA expression of various TLRs and their co-receptors may predispose monocytes to increased TLR-mediated activation in chronic HCV.

Lymphocytes of HCV-infected patients express higher levels of TLRs compared to controls

We next investigated the expression of TLRs in CD3+ T cells of the adaptive immune cells. T cells from controls and HCV infected patients expressed mRNA for TLRs1-10 (Figure 3) as expected based on previous publications^[14,15]. There was no CD14 mRNA expressed in T lymphocytes of either controls or HCV-infected patients, confirming the purity of the cell population (data not shown). We found that levels of RNA coding for membrane-localized TLR1, 2, 4, 6 and 10 as well as the TLR4 co-receptor, MD2, were significantly higher in HCV infected patients compared to controls. No differences in levels of RNA coding for MyD88 or TLR3 and TLR5 (Figure 3) between controls and patients were identified. In contrast, the RNA levels of intracellularly localized TLRs 7, 8 and 9 were significantly higher in T lymphocytes of HCV infected patients compared to controls.

DISCUSSION

Monocytes and lymphocytes, respectively, are representative populations of innate and adaptive immunity and their cooperation is required to recognize, limit and eliminate invading microbes^[20]. Here we show that patients infected with HCV have a unique pattern of TLR expression in

both the innate and adaptive immune compartments and have elevated RNA expression of both membrane-associated and intracellularly localized TLRs. Recent reports demonstrate that HCV-infected patients have a higher prevalence of infections with cytomegalovirus, Cryptococcus, Mycobacterium tuberculosis and sexually transmitted diseases compared to controls^[5]. Recognition of these pathogens requires TLR-mediated signals. For example, sensing of Cryptococcus by the innate immune system requires TLR2, TLR4, MyD88 and CD14^[1], while TLR2 and TLR4 are instrumental in the host's response against Mycobacteria^[21]. The pathogens implicated in sexually-transmitted diseases, found more often in HCV-infected patients, are also recognized by TLR. Chlamydia elicits an unusual set of inflammatory responses via TLR2 and TLR4 *in vivo*, and TLR2 is essential for development of oviduct pathology in chlamydial genital tract infection^[22], while *Neisseria gonorrhoeae* stimulates cytokine release and NF- κ B activation in epithelial cells in a TLR2-dependent manner^[23]. In addition, some TLRs may play a protective or even therapeutic role in defense against sexual disease pathogens: TLR3 agonists protect against genital herpes infection^[24] and Imiquimod, a TLR7&8 ligand and the first FDA-approved imidazoquinoline, has been approved for the therapy of genital warts^[25]. Increased expression of TLR2 and TLR4 is a bad prognostic factor in patients with sepsis^[26], while low TLR expression may protect the host against excessive inflammation and tissue damages^[27], thus, differential expression of TLRs in HCV infected patients may contribute to susceptibility to infections as well as to the underlying disease progression and this remains to be determined.

The observation of increased expression of TLR2 in patients was not totally unexpected, since chronic HCV infection is associated with elevated levels of circulating LPS, which upregulates the expression of TLR2 in different cell types, including monocytes^[1,21,22]. We have previously shown that HCV core and non-structural protein 3 (NS3), activate cells through TLR2^[10]. We found elevated levels of the TLR co-receptors, CD14 and MD2, and adaptor molecule MyD88, in HCV-infected patients compared to controls, suggesting that MyD88-mediated TLR signal transduction may also be affected in patients. Indeed, we have previously found that monocytes from HCV infected patients are hyper-responsive to stimulation with TLR4 and TLR2 ligands^[10,31]. Although we did not find a role for CD14 in TLR2-mediated cell activation by HCV-derived core and NS3 proteins (unpublished data), increased CD14 expression may be relevant to cell activation mediated by other TLR2 and TLR4 ligands, as previously reported for LPS, lipoteichoic acid and even viruses^[9].

Intracellularly-localized TLR 3, 7, 8 and 9 are of specific interest in patients with chronic viral infections since these receptors can recognize virus-derived molecular patterns^[6,7]. We found that TLR3 expression was higher in monocytes of HCV-infected patients compared to controls. TLR3 is a sensor for double-stranded RNA and it has been implicated in pathogenesis of viral infections^[32,33]. Synthetic RNA compounds, such as polyI:C activate cells expressing TLR3^[34]. In a recent study by Edelman *et al.*^[35] the role of TLR3 was investigated in four differ-

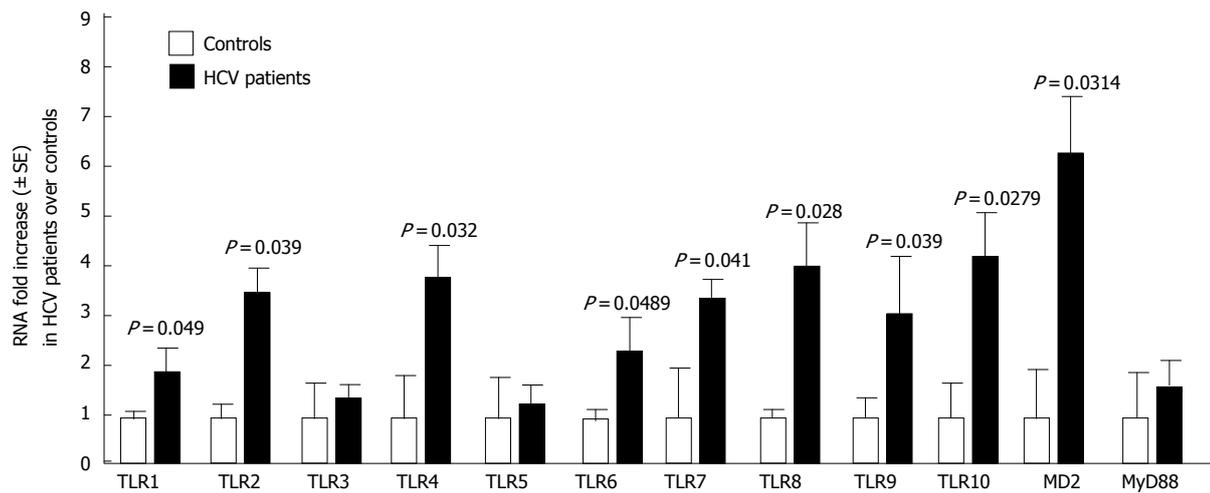


Figure 3 Higher TLRs 1, 2, 4, 6, 7, 8, 9 and 10 and MD2 RNA expression by lymphocytes of HCV-infected patients compared to controls. RNA was isolated from lymphocytes and real-time PCR for TLRs 1-10, MD2 and 18S RNA (as endogenous control) was performed as described in Materials and Methods. For each individual, TLR- or MD2-coding RNA expression was normalized to corresponding 18S RNA. Results are expressed as fold increase of each TLR or MD2 in HCV-infected patients compared to controls. Results are from 14 HCV-infected patients and 14 controls.

ent infectious viral models [lymphocytic choriomeningitis virus (LCMV), vesicular stomatitis virus (VSV), murine cytomegalovirus (MCMV), and reovirus in TLR3^{-/-} mice]. The investigators found that TLR3 is not always required for the generation of effective antiviral responses, as the absence of TLR3 did not alter either viral pathogenesis or host's generation of adaptive antiviral responses to those viruses. Interestingly, intracellular transduction of poly(I:C) initiates activation of an IFN response in a TLR3-independent manner, thus limiting the role of TLR3 in the IFN pathway^[54]. A recent report by Li *et al*^[53] indicates that HCV may use TLR3 pathway to evade immune surveillance via HCV NS3/4A protease-mediated cleavage of the TLR3 adaptor protein TRIF. Furthermore, HCV NS3/4A interferes with retinoic acid-inducible gene-I (RIG-I), a key factor in TRIF-independent signaling^[54]. Our finding of increased TLR3 mRNA levels in monocytes of HCV patients awaits further investigation. It is tempting to speculate that HCV RNA may trigger cellular activation via TLR3, and indeed, all our patients had high levels of viremia, however no clear relationship between HCV RNA levels and TLR3 expression has been proven to date.

TLR7 and TLR8 are stimulated by synthetic compounds imiquimod and resiquimod 848^[6,7,54]. In a recent publication, Lund *et al*^[12] showed that TLR7 recognizes the single-stranded RNA viruses, vesicular stomatitis virus and influenza virus. The recognition of these viruses by plasmacytoid dendritic cells and B cells through TLR7 results in cellular activation and the production of cytokines. However, the specific role of TLR7 and TLR8 in innate immune response to HCV is yet to be understood. HCV is a single stranded RNA virus, thus, it could theoretically act as a ligand for TLR7. Our data show that TLR7 mRNA is significantly upregulated in HCV patients compared to controls, and all patients had detectable levels of HCV RNA in their plasma, thus suggesting that TLR7 may be implicated in the pathogenesis of HCV infection.

We found that both monocytes and T cells of HCV-infected patients expressed elevated levels of TLR9

mRNA compared to controls. CpG-containing DNA acts as a stimulatory ligand for TLR9^[6,7,28,5]. TLR9 is regulated at the transcriptional level by multiple nuclear regulatory factors, co-repressors and co-activators, including CREB1, Ets2, Elf1, Elk1, and C/EBP and HCV may regulate these transcription factors^[56,57]. Toll-like receptors 9 and 3 are essential components of innate immune defense against cytomegalovirus infection in mice^[58]. While we found that TLR9 mRNA was upregulated in patients, others have shown that infections with cytomegalovirus are more frequent in HCV-infected patients compared to controls^[5]. It remains to be elucidated if the increased TLR9 RNA levels in monocytes or T cells have a role in increased susceptibility to infections with CMV, as seen in HCV patients.

To date little is known about the role of TLR expression in T cells of the adaptive immune compartment. Caramalho *et al*^[39] recently reported that LPS, a TLR4 ligand, promotes survival of activated CD4⁺ cells and stimulates regulatory T cell (T reg) activity. Here we found that TLR4 mRNA expression was higher in HCV-infected patients compared to controls only in lymphocytes, but not in monocytes. It is tempting to speculate that elevated levels of TLR4 and its co-receptor, MD-2, in patients' T cells may provide ongoing activation in the presence of serum LPS found in chronic HCV and provide preferential survival signals for T regs^[29]. Recent data suggest a role for T regs in immune alterations associated with chronic HCV. Cabrera *et al*^[40] reported that Tregs appear to play a role in viral persistence by suppressing HCV-specific T cell responses in a cell-cell contact manner, while MacDonald *et al*^[41] showed that Tregs could be induced against the same epitopes on the HCV core protein.

A limitation of our study is that it did not distinguish between TLR expression in CD4⁺ and CD8⁺ T cells. Differential CD4⁺ and CD8⁺ T-cell responsiveness in hepatitis C virus infection has been reported, thus, leading to the hypothesis that if TLRs are of any functional role in T cells, there may be differences in TLR expression between CD4⁺ and CD8⁺ T cells in HCV-infected patients. Due to

the presence of anti-CD56 mAbs in the separation cocktail, we also lost CD56⁺ T cells, known as gamma delta, with an important role in HCV infection^[42].

In conclusion, we showed that patients with chronic HCV infection express elevated levels of selected TLRs in both adaptive and innate compartments of the immune system. Our data may suggest additional therapeutic targets.

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Autoimmune thrombocytopenia in response to splenectomy in cirrhotic patients with accompanying hepatitis C

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Abstract

AIM: To estimate the contribution of autoimmune thrombocytopenia to hepatitis C virus-related liver cirrhosis (type C cirrhosis), we evaluated the influence of splenectomy upon platelet-associated immunoglobulin G (PAIgG) levels and platelet numbers.

METHODS: PAIgG titers and immune markers were determined in 24 type C cirrhotic patients with an intact spleen, 17 type C cirrhotic patients submitted to splenectomy, and 21 non-C cirrhosis with an intact spleen.

RESULTS: Thrombocytopenia ($PLT < 15 \times 10^4 / \mu L$) in type C cirrhosis was diagnosed in all patients with an intact spleen, 8 patients submitted to splenectomy, and in 19 non-C cirrhosis with intact spleen. Elevated titers of PAIgG at more than 25.0 ng/ 10^7 cells were detected in all cirrhotic patients except for one splenectomized patient. PAIgG titers (ng/ 10^7 cells) were significantly higher in the type C cirrhosis with an intact spleen (247.9 ± 197.0) compared with the splenectomized patients (125.6 ± 87.8) or non-C cirrhosis (152.4 ± 127.4). PAIgG titers were negatively correlated with platelet counts in type C cirrhotic patients with an intact spleen. In comparison with the type C cirrhosis with an intact spleen, the splenectomized patients had a reduced CD4/CD8 ratio and serum neopterin levels. The spleen index (cm^2) was negatively correlated with platelet counts in the non-C cirrhosis, but not in the type C cirrhosis.

CONCLUSION: Our data indicate that the autoimmune mechanism plays an important role in thrombocytopenia complicated by HCV-positive cirrhosis. In addition, splenectomy may impair T cells function through, at least in part, a reduction of CD4/CD8 ratio, consequently suppressing PAIgG production.

INTRODUCTION

Accompanying thrombocytopenia in patients with liver cirrhosis was initially attributed to an increase in platelet sequestration in the enlarged spleen secondary to portal hypertension^[1]. Elevated platelet counts after procedures, such as transjugular intrahepatic portosystemic shunt^[2,3], partial splenic embolization^[4,5], splenectomy^[6], and shunt operation^[7,8], confirmed this theory. Nevertheless, controversial results on the relief of portal hypertension by surgical means have shown a reduction in spleen size with no concomitant persistent rise in platelet counts unless accompanied by liver transplantation^[9]. Thus, the clinical significance of portal hypertension in the pathogenesis of thrombocytopenia in liver cirrhosis remains to be elucidated. Recently, not only impaired production of thrombopoietin, which regulates megakaryocyte development^[10,11], but also immune disturbance is thought to be responsible for thrombocytopenia associated with cirrhosis. In autoimmune-mediated thrombocytopenia, patients with chronic liver disease had a high incidence of platelet-associated immunoglobulin G (PAIgG)^[12-16]. Pereira *et al*^[12] detected autoantibodies that reacted specifically with platelet membrane glycoprotein in chronic liver disease, suggesting that an immune mechanism may participate in the induction or aggravation of thrombocytopenia.

Hepatitis C virus (HCV) has been identified as a major causative agent for parenterally transmitted non-A non-B hepatitis throughout the world^[17]. Approximately 85% of HCV-infected individuals fail to clear the virus and develop persistent chronic hepatitis which leads to cirrhosis^[18]. Chronic HCV infection is associated with an increased risk of liver cancer, and most cases of HCV-

related hepatocellular carcinoma occur in the presence of cirrhosis. In addition, HCV infection is often associated with immune disturbance, including the development of autoantibodies and an increased frequency of immune-mediated disease^[19]. We already hypothesized that chronic HCV infection may cause autoimmune thrombocytopenia because of prevalence of elevated levels of PAIgG and an inverse correlation between PAIgG titers and platelet counts^[20]. On the other hand, Kosugi *et al*^[21] reported that hepatitis C patients had a high incidence of PAIgM but relatively low levels of PAIgG, insisting that thrombocytopenia in hepatitis C patients was not due to anti-platelet autoantibodies. Substantial evidence exists that PAIgG is frequently identified in HCV-positive patients^[20,22], but whether this immunoglobulin acts as a specific anti-platelet antibody remains unclear.

The role of spleen in PAIgG production and modification of cell-mediated immunity was previously reported in patients with idiopathic thrombocytic purpura (ITP), in whom the prevalence of PAIgG was found to contribute to the development of autoimmune thrombocytopenia^[23]. Chronic ITP patients characteristically had varied PAIgG levels post-splenectomy^[24], but showed immediate normalization in splenectomy-responders^[25]. In ITP patients, CD4+ and HLA-DR-restricted T cells to platelet membrane GPIIb-IIIa were found to involve in the production of anti-platelet autoantibodies^[26]. These results suggest that splenectomy may modify PAIgG production and immune mediators in patients with chronic HCV infection. In the present study, we, in order to elucidate the role of autoimmune thrombocytopenia in the development of HCV-positive liver cirrhosis, retrospectively evaluated the influence of splenectomy on PAIgG levels and platelet counts, as well as cellular and humoral immune mediators.

MATERIALS AND METHODS

Patients

In the present study, 41 patients with type C cirrhosis, who were referred to our hospital, were enrolled. They were found to be seropositive for HCV antibodies detected a second or third generation enzyme-linked immunosorbent assay (Ortho Diagnostic System Co., Ltd., Tokyo, Japan) and/or recombinant immunoblot assay (Chiron RIBA-2 and/or RIBA-3). Seventeen patients had undergone splenectomy in association with surgery for esophageal varices (the splenectomies). The mean duration of follow-up after splenectomy was 11.5 ± 4.7 years (range, 2 to 19 years). Twenty-four consecutive cirrhotic patients had an intact spleen (the non-splenectomies). In addition, 20 cirrhotic patients including 10 hepatitis B, 4 autoimmune hepatitis, 3 alcoholic and 3 idiopathic cirrhosis served as the non-C cirrhosis. All the non-splenectomies and non-C cirrhosis had esophageal varices complication, and eight of the non-splenectomies and five of the non-C cirrhosis received treatment for the varices by endoscopic sclerotherapy and/or band ligation.

Morphological diagnosis of cirrhosis was performed in all the splenectomies, 15 non-splenectomies and 12 non-C cirrhosis. The others were diagnosed clinically, and they had typical laboratory findings of liver cirrhosis and

Table 1 Characteristics of the patients (n, mean±SD)

| | Type C cirrhosis | Type C cirrhosis submitted to splenectomy | Non-C cirrhosis |
|------------------------|------------------|---|-----------------|
| Number | 17 | 24 | 20 |
| Age (yr) | 62.6±10.6 | 63.0±7.1 | 61.8±9.5 |
| Gender | | | |
| Female | 10 | 15 | 10 |
| Male | 7 | 9 | 10 |
| Child's classification | | | |
| A | 11 | 13 | 9 |
| B | 4 | 6 | 7 |
| C | 2 | 4 | 4 |
| Association of HCC | 4 | 7 | 5 |

Non-C cirrhosis consisted of 10 hepatitis B, 4 autoimmune hepatitis, 3 alcoholic and 3 idiopathic cirrhosis. HCC: hepatocellular carcinoma.

characteristic liver imaging on computed tomography or ultrasonography. Patients' characteristics are shown in Table 1. Clinical features were similar among the three groups. Informed consent was obtained from each patient according to the Helsinki Declaration.

Assays

Quantitative HCV-RNA was analyzed from serum samples by a combined reverse transcription PCR assay (Amplicor-HCV monitor). Peripheral blood counts and liver function parameters were measured by an autoanalyzer.

Quantity of PAIgG was determined by a competitive micro enzyme-linked immunosorbent assay (ELISA) as described by Kawaguchi *et al*^[27]. Briefly, platelets were separated from whole blood collected in EDTA, and washed with phosphate-buffered saline containing 11 g/L bovine serum albumin. The ELISA was performed in 96-well microplates coated with purified human IgG. A horseradish peroxidase-conjugated anti-human IgG antibody was incubated simultaneously with the samples, and visualized with o-phenylenediamine. PAIgG titers from 49 healthy individuals were within $25.0 \text{ ng}/10^7$ cells (mean, $16.4 \pm 4.2 \text{ ng}/10^7$ cells)^[20].

The proportion of CD4 and CD8 subsets of peripheral bloods was assayed by flow cytometry. The levels of neopterin and soluble interleukin-2 receptor (sIL2R) were measured using HPLC and Cell Free IL-2R EIA kit, respectively. Immunological markers and PAIgG were determined using fresh blood samples.

Spleen size in cirrhotic patients was evaluated as spleen index using ultrasonography as previously reported^[28], in which spleen index correlated well with the volumes of resected spleens. Briefly, the spleen index was calculated as the product of the transverse diameter and its perpendicular diameter measured on the maximum cross-sectional image of the spleen, and expressed in cm^2 . Values obtained from healthy individuals were below 20 cm^2 , and the mean values of 28 healthy controls were $15 \pm 7 \text{ cm}^2$ ^[28].

Statistical analysis

Values were expressed as mean±SD. Comparisons of the serum measurements among patients of the splenectomies, non-splenectomies and non-C cirrhosis were carried out using analysis of variance (ANOVA), and analyses of

Table 2 Laboratory findings in the type C cirrhotic and non-C cirrhotic patients (*n*, mean±SD)

| | Type C cirrhosis | Type C cirrhosis submitted to splenectomy | Non-C cirrhosis |
|----------------------------------|------------------|---|-----------------|
| RBC (×10 ⁴ /μL) | 379±61 | 374±65 | 381±63 |
| WBC (/μL) | 3 652±1 080 | 5 855±1 595 ¹ | 3 832±1 048 |
| Platelet (×10 ⁴ /μL) | 8.2±2.9 | 16.1±4.9 ¹ | 9.2±3.4 |
| >15.0 | 0 | 9 | 1 |
| 10.0-14.9 | 5 | 6 | 6 |
| <9.9 | 19 | 2 | 13 |
| ALT (IU/L) | 58.2±25.3 | 51.1±35.2 | 40.6±23.8 |
| Total bilirubin (mg/dL) | 1.4±1.2 | 0.9±0.3 ¹ | 2.3±1.8 |
| Albumin (g/L) | 37±4 | 37±6 | 34±8 |
| r-globulin (g/L) | 21±0.5 | 22±6 | 20±9 |
| IgG (mg/ dL) | 2 454±693 | no assay | 2 220±877 |
| HCV RNA (kcopies/mL) | 523±403 | 198±251 ² | |
| Splenic index (cm ³) | | | |
| - 19 | 6 cases | | 5 cases |
| 20 - 29 | 11 cases | | 9 cases |
| 30 - 39 | 6 cases | | 5 cases |
| 40 - | 1 case | | 3 cases |

ALT: alanine aminotransferase. 1: Significant difference compared with the type C cirrhosis with an intact spleen and non-C cirrhosis. 2: Significant difference compared with the type C cirrhosis with an intact spleen.

Table 3 Immunological markers in patients with type C cirrhosis (mean±SD)

| | Type C cirrhosis | Type C cirrhosis submitted to splenectomy |
|---------------------|------------------|---|
| Neopterin (pmol/mL) | 8.3±3.2 | 5.2±2.5 ^b |
| sIL2R (U/mL) | 850.7±175.9 | 877.2±458.6 |
| CD4 (%) | 43.1±8.6 | 33.4±7.6 |
| CD8 (%) | 27.6±7.9 | 29.6±9.6 |
| CD4/CD8 ratio | 1.8±0.9 | 1.3±0.4 ^a |

sIL2R: Soluble interleukin-2 receptor. ^a*P*<0.05 *vs* type C cirrhosis with an intact spleen; ^b*P*<0.01 *vs* type C cirrhosis with an intact spleen.

non-parametric data between two groups were carried out by Mann-Whitney *U* test. A correlation was calculated with the Spearman's rank correlation coefficient.

RESULTS

Laboratory findings and spleen index

Thrombocytopenia (PLT<15×10⁴/μL) was diagnosed in all the non-splenectomies (100%), eight of the splenectomies (47.1%), and 19 of the non-C cirrhosis (95.0%) (Table 2). Marked thrombocytopenia at less than 10×10⁴/μL was obviously found in the non-splenectomies (79.2%) and the non-C cirrhosis (65.0%) as compared with the splenectomies (11.8%). Peripheral platelet and white blood cells were significantly higher in the splenectomies than that in the non-splenectomies and non-C cirrhosis (*P*<0.01). Platelets and white blood cells were similar in the non-splenectomies and non-C cirrhosis. Red blood cell counts were not changed among the three groups. No difference was found among the three groups for liver function parameters except for total bilirubin levels and HCV-RNA titers.

Spleen index ranged widely from 9.6 to 42.0 cm³. The

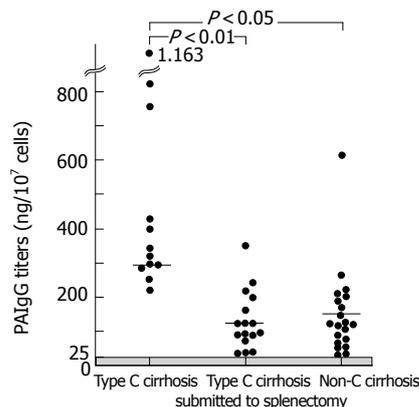


Figure 1 PAIgG titers in the type C cirrhosis with an intact spleen, type C cirrhosis submitted to splenomegaly, and non-C cirrhosis. The type C cirrhosis with an intact spleen had significant higher titers of PAIgG as compared with those of the splenectomized patients with type C cirrhosis and non-C cirrhosis.

mean spleen index was above the upper limit of normal individuals (20 cm³) in both type C cirrhosis (24.6±8.0 cm³) and non-C cirrhosis (23.5±8.0 cm³).

Immunological markers

An elevation of PAIgG at more than 25.0 ng/10⁷ cells was observed in all cirrhotic patients except for one splenectomized patient. The mean PAIgG titers were significantly higher in the non-splenectomies as compared with the splenectomies (297.9±197.0 *vs* 125.6±87.8 ng/10⁷ cells, *P*<0.05) and non-C cirrhosis (297.9±197.0 *vs* 152.4±127.4 ng/10⁷ cells, *P*<0.05). There was no significant difference in PAIgG titers between the splenectomies and the non-C cirrhosis (Figure 1). In comparison with the type C cirrhotic patients, the splenectomies had reduced percentage of CD4 and similar percentage of CD8, resulting in a lower ratio of CD4/CD8 than those of the non-splenectomies. In addition, neopterin levels were significantly lower in the splenectomies as compared with the non-splenectomies, while sIL2R levels were similar between the two groups (Table 3).

Correlation between peripheral blood cells, immunological markers and spleen index

In the non-splenectomies, PAIgG titers were negatively correlated with platelet counts, and positively correlated with IgG and γ-globulin levels (Figure 2). In the non-C cirrhosis, PAIgG titers were neither correlated with platelet counts nor with IgG and γ-globulin levels. A significant negative correlation between the spleen index and platelet counts was found in the non-C cirrhosis, whereas not in the type C cirrhosis (Figure 3). In addition, the spleen index was negatively correlated with white blood cell counts in both type C and non-C cirrhosis. In the splenectomies, PAIgG titers were not correlated with platelet counts, γ-globulin levels, or the duration of follow-up after splenectomy.

DISCUSSION

Our data demonstrated that the HCV-positive cirrhosis (type C cirrhosis) with an intact spleen had a significant rise

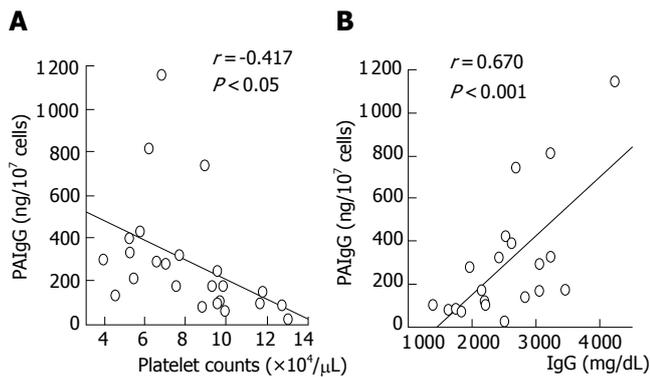


Figure 2 Relationships among PAIgG titers, platelet counts, and IgG levels in the type C cirrhosis with an intact spleen. PAIgG titers are negatively correlated with platelet counts, and positively correlated with IgG levels.

of PAIgG as compared with the non-C cirrhosis, including hepatitis B infection, autoimmune hepatitis, alcohol abuse, and idiopathic cirrhosis. PAIgG titers were negatively correlated with platelet counts in the type C cirrhosis with an intact spleen. The type C cirrhotic patients submitted to splenectomy showed a significant elevation of platelet counts and reduction in PAIgG titers compared with those of the patients with an intact spleen. Similar PAIgG and platelet levels following splenectomy are commonly found in ITP patients, in whom the thrombocytopenia is responsible for the autoimmune mechanism mediated by a specific IgG bound to platelet membrane proteins^[24,25]. There exist controversies regarding the clinical significance of PAIgG in pathogenesis of thrombocytopenia in the patients with liver disease^[7,14,29,30]. Two studies on partial splenic artery embolization in patients with hypersplenism clearly confirmed an immunological mechanism mediated by PAIgG-induced thrombocytopenia accompanying liver cirrhosis^[7,14]. These studies reported a significant rise in platelet numbers and a significant fall in PAIgG levels after partial splenic artery embolization. In the present study, the changes in PAIgG levels and platelet numbers among the type C cirrhotic patients with or without an intact spleen are practically similar to those previous results. These data support a key role of spleen in PAIgG production and that an autoimmune mechanism plays an important role in the development of thrombocytopenia associated with HCV-positive cirrhosis. On the contrary, a non-specific adsorption of elevated γ -globulin by platelets was suspiciously reported on chronic liver disease^[27]. Our data found a negative correlation between PAIgG titers and platelet counts in the type C cirrhosis with an intact spleen, but not in the non-C cirrhosis. Presumably, the elevated PAIgG in the type C cirrhosis may act as anti-platelet autoantibodies. However, we found a positive correlation between PAIgG titers and IgG levels in the type C cirrhosis with an intact spleen. This result is in agreement with that of de Noronha *et al*^[31] in the patients with various liver diseases. The methods used for quantitating PAIgG in their study as well as in our study appear to measure total PAIgG, which is not equivalent to platelet-specific autoantibodies. They suggested that increased platelet IgG was due to enhanced retention of plasma IgG in the platelets.

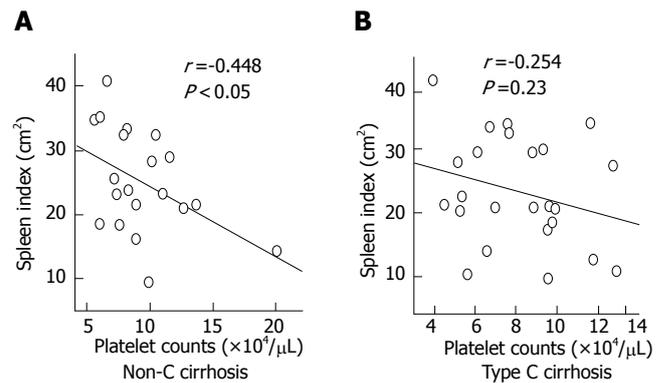


Figure 3 Relationship between the spleen index and platelet counts. A significant negative correlation is shown in the patients with non-C cirrhosis, but not in the type C cirrhosis with an intact spleen.

In our study, despite similar γ -globulin levels, a relationship between PAIgG and γ -globulin was found neither in the splenectomized patients with type C cirrhosis nor in the non-C cirrhotic patients. Consequently, the theory of 'enhanced retention of IgG in the platelets' proposed by de Noronha *et al*^[31] is deniable in the present study.

Sampele *et al*^[32] indicated that CD4+ T-helper cells from patients with ITP are stimulated by normal platelet antigen(s) to secrete IL-2 and may modulate the enhanced anti-platelet autoantibody response. The underlying mechanisms for the production of anti-platelet autoantibodies require CD4+ and HLA-DR-restricted T cells to GPIIb-IIIa in ITP patients^[26]. GPIIb+ cells isolated from spleens of patients with chronic ITP strongly expressed HLA-DR, and splenic T cells had a high level of *in vitro* platelet-stimulated IL-2 secretion as compared with the controls^[33]. Thus, the spleen is such an important site for antibody production in patients with chronic ITP that the levels of PAIgG rapidly normalize in response to a splenectomy^[24,34]. Our data showed that compared with the type C cirrhosis with an intact spleen, the type C cirrhosis submitted to splenectomy had lower CD4/CD8 ratio because of a reduced percentage of CD4+ T cells. Neopterin is secreted by macrophages when stimulated for helper functions in the proliferation and activation of T cells. This immune marker was significantly decreased in the type C cirrhosis submitted to splenectomy compared to the type C cirrhotic patients with an intact spleen, supporting impaired T cell function following splenectomy. Taken together, previous and present results indicate that splenectomy may impair T cell function in HCV-positive liver cirrhosis through, at least in part, a reduction in CD4/CD8 ratio, which thereby suppresses PAIgG production. The data from the present study provide some indications that the spleen induces helper T-cell proliferation, but a further research is necessary to clarify the role of spleen in PAIgG production and T helper lymphocyte cytokine production.

Hypersplenism due to portal hypertension is believed as a major cause of thrombocytopenia complicated with liver cirrhosis; however, studies on the relief of portal hypertension have yielded the controversial results that observable reduction in spleen size is not associated with a concomitant persistent rise in platelet counts, unless accom-

panied by liver transplantation^[8,9]. Thus, the role played by immune disturbance as well as thrombopoietin is an attractive possibility in the pathogenesis of thrombocytopenia concomitant with liver cirrhosis. Noguchi *et al*^[5] observed the improved numbers and survival time of platelets and reduction in the raised PAIgG levels by partial splenic artery embolization in cirrhotic patients with hypersplenism. Owing to a negative correlation between platelet numbers and splenic volume before and after the procedure, they concluded that partial splenic artery embolization may not only reduce the increased platelet pool in the spleen, but also improve the autoimmune thrombocytopenia induced by cirrhosis. In the present study, the elevated platelets and reduced PAIgG levels in the splenectomized patients support their results. In addition, the present study showed that spleen size was correlated with platelets counts in the non-C cirrhotic patients. The data suggested that hypersplenism due to portal hypertension is assumed to be a major cause of thrombocytopenia observed in the non-C cirrhotic patients. If no significant relationship is found between the spleen index and platelet counts in HCV-positive cirrhotic patients, autoimmune disturbance may be the most likely principal mechanism for thrombocytopenia rather than sequestration and/or destruction of platelets secondary to hypersplenism.

In the present retrospective study, we confirmed that the autoimmune disturbance induces thrombocytopenia associated with liver cirrhosis which is related to HCV-infection. Prospective pre- and post-splenectomy studies should elucidate the role of spleen in PAIgG production, platelet sequestration and destruction, as well as cellular and humoral immunity.

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Smad3 knock-out mice as a useful model to study intestinal fibrogenesis

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in null mice as compared to wild-type mice.

CONCLUSION: Smad3 null mice are a useful model to investigate the *in vivo* role of the TGF- β /Smad signalling pathway in intestinal inflammation and fibrosis.

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Key words: Transforming growth factor; TGF- β ; Fibrosis; Smad proteins

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Abstract

AIM: To evaluate the possible differences in morphology and immunohistochemical expression of CD3, transforming growth factor β 1(TGF- β 1), Smad7, α -smooth muscle actin (α -Sma), and collagen types I-VII of small and large intestine in Smad3 null and wild-type mice.

METHODS: Ten null and ten wild-type adult mice were sacrificed at 4 mo of age and the organs (esophagus, small and large bowel, ureters) were collected for histology(hematoxylin and eosin, Masson trichrome, silver staining), morphometry and immunohistochemistry analysis. TGF- β 1 levels of intestinal tissue homogenates were assessed by ELISA.

RESULTS: No macroscopic intestinal lesions were detected both in null and wild-type mice. Histological and morphometric evaluation revealed a significant reduction in muscle layer thickness of small and large intestine in null mice as compared to wild-type mice. Immunohistochemistry evaluation showed a significant increase of CD3+T cell, TGF- β 1 and Smad7 staining in the small and large intestine mucosa of Smad3 null mice as compared to wild-type mice. α -Sma and collagen I-VII staining of small and large intestine did not differ between the two groups of mice. TGF- β 1 levels of colonic tissue homogenates were significantly higher in null mice than in wild-type mice. In preliminary experiments a significant reduction of TNBS-induced intestinal fibrosis was observed

INTRODUCTION

Smads are a family of eight related proteins which function as signalling intermediates for the transforming growth factor β (TGF- β) superfamily of ligands^[1-3]. Upon ligation and activation of TGF- β with its receptors (I, II and III), the phosphorylated Smad2 and 3 form a complex with the common mediator Smad4. The Smad2/3- Smad4 complex can translocate into the nuclei where they enhance specific TGF- β target genes. The inhibitory Smad7 antagonizes TGF- β signalling by interfering with the ligation of Smad2/3 with the activated receptor complex. Experimental evidence from several research groups suggests that disruption of the TGF- β /Smad signalling pathway plays a central role in sustaining both chronic tissue inflammation and fibrosis^[4-6].

TGF- β is a multifunctional polypeptide hormone influencing different functions in a variety of cells including regulation of cell proliferation, differentiation and apoptosis, immunoregulation, regulation of the inflammatory response, restitution and healing^[5,7,8]. At cellular level, TGF- β affects virtually all stages of the chronic inflammatory and fibrotic disease processes. The effects of TGF- β on the extracellular matrix are more complex than those of any other growth factor and are central to its effects in increasing the maturation and strength of wounds, as well as in the pathological matrix accumulation, characteristic of fibrotic disease^[4,9,10]. TGF- β regulates the transcription

of a wide spectrum of matrix proteins including collagen, fibronectin, glycosaminoglycans, and matrix-degrading proteases (metalloproteinases) and their inhibitors.

TGF- β /Smad signalling plays an important role in chronic inflammatory diseases, especially in Crohn's disease (CD)^[5,7,11,12]. The transmural infiltrate of CD is responsible for initiating and maintaining a series of connective tissue changes not only involving the mucosa but also the submucosa and *muscularis mucosae* and *propria* where a marked increase of type I, III and V collagens and RNA transcripts are observed^[13-15]. In CD, there is a marked overexpression of TGF- β 1 and TGF- β receptors in the colonic mucosa^[16-18]. Fibrosis in CD can therefore be viewed as an aberrant healing response to injury^[19]. In addition, TGF- β appears to be involved in intestinal fibrosis present in other enteropathies, such as radiation enteritis, collagenous colitis and intestinal graft-versus-host disease^[20-22].

Experimental transgenic animal models are useful tools to study the *in vivo* function of individual molecules^[23-25]. TGF- β knock-out mouse model is characterized by the loss of a critical regulator of immune function which leads to an excessive inflammatory response with massive infiltration of leukocytes in several organs^[26]. This condition develops rapidly with onset during the first week of life and results in severe wasting and death by the fourth week of life^[27,28]. Unlike the targeted disruptions of Smad2 and 4 which are lethal^[29,30], the disruption of Smad3^[31] results in the birth of mice which are viable and can survive to adulthood (up to 8 mo of age). Since the Smad3 knock out model provides pivotal information concerning cutaneous wound healing^[32-34], it is thought that this model might also be useful to investigate the *in vivo* role of the TGF β /Smad signalling pathway in intestinal inflammation and fibrosis.

The present study was to evaluate the small bowel and colonic morphology as well as the immunohistochemical expression of collagens I-VII, α -smooth muscle actin (α -SMA), TGF β 1, Smad7, and CD3 in Smad3 wild-type and null mice.

MATERIALS AND METHODS

Animals

Colonies of Smad3 wild-type, heterozygous and null mice (black Swiss strain) were bred in our laboratory. These animal colonies were developed using pairs of Smad3 heterozygous mice kindly provided by A. Roberts (NCI, Bethesda, MD, USA). Smad3^{ex8/ex8} mice were generated by targeted disruption of the Smad3 gene by homologous recombination. Targeted embryonic stem-cell clones were injected into a C57BL blastocyst to obtain germline transmission. Mice heterozygous for the targeted disruption were intercrossed to produce homozygous offspring^[31].

All mice were maintained in a specific pathogen-free (SPF) facility and routinely monitored. Mice were kept in microisolator cages and allowed free access to food and water. All mice were examined 4 times a week for signs of colitis including weight loss, diarrhea, rectal bleeding and prolapse^[35], as well as signs of systemic inflammation such as piloerection, lethargy and periorbital exudates^[36].

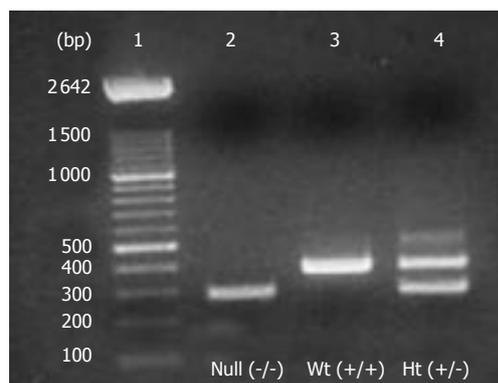


Figure 1 Genotyping of animal offsprings by PCR of cDNA (tail extracts). Lane 1= molecular weight ladder of 100 bp; lane 2= null mice; lane 3= wild-type (Wt) mice; lane 4= heterozygous(Ht) mice.

DNA extraction and genotype analysis

Mouse tail DNA extraction was performed according to the protocol reported elsewhere^[37].

Genotype analysis was carried out by the polymerase chain reaction (PCR) method in which the wild type Smad3 allele was detected using primer 1 (5'-CCAGACTGCCTTGGGAAAAGC-3') and primer 2 (5'-CCCGAACAGTTGATTCACACA-3'). Primer 1 is located 5' to the deletion and primer 2 is located within the deletion. This primer pair amplified a fragment of ~ 400 bp from wild-type and Smad3^{ex8/ex8} mice, but not from Smad3^{ex8/ex8} mice (Figure 1). DNA was also amplified using primer 1 and primer 3 (5'-CCAGACTGCCCTTGGGATGCCCTG-3'), which is located in the pLoxpneo, to detect the mutant Smad3 allele. In this case, a 250 bp fragment was detected in mice, heterozygous or homozygous for the mutant Smad3 allele, while no signal was detected in wild-type mice.

Sample recovery and preparation

A total of 20 mice (10 wild-type and 10 null for the Smad3 allele) were sacrificed at 4 mo of age. Laparotomy was performed under ether anesthesia. The esophagus, small bowel, colon and ureters were visualized, rapidly excised and placed in a Petri dish containing sterile saline solution. Tissue samples from the esophagus, small bowel, colon, and ureters were processed for structural and immunohistochemical studies.

Histology and morphometry

Specimens obtained from the esophagus, small bowel, colon and ureters of all animals were washed and immediately immersed in 10% buffered formalin in phosphate buffer saline (PBS) (pH 7.4) for 3 h at room temperature followed by the standard procedure for paraffin embedding. Serial 3 μ m sections were stained with hematoxylin and eosin (HE) to assess the degree of inflammation and with Masson trichrome and silver stain to detect connective tissue. The stained sections were then observed under an Olympus BX51 light microscope (Olympus, Optical Co. Ltd., Tokyo, Japan). The degree of intestinal inflammation was scored as absent, mild, moderate or severe according to the density and extent of both acute and chronic inflammatory infiltrate, loss of goblet cells and

Table 1 Muscle layer thickness of gastrointestinal and urinary tract segments from Smad3 wild-type and null mice (mean±SD)

| | Wild-type (n) | Null (n) | P value |
|-------------|------------------|------------------|---------|
| Colon | 221.79±99.78 (6) | 104.68±50.73 (7) | 0.02 |
| Small bowel | 95.55±38.45 (6) | 46.44±25.96 (6) | 0.03 |
| Esophagus | 189.36±42.60 (5) | 188.49±43.94 (6) | NS |
| Ureters | 32.85±3.74 (5) | 30.31±1.91 (5) | NS |

(n)=Number of mice evaluated. Muscle layer thickness is expressed in micron. NS=not significant.

bowel wall thickening^[35,38]. Intestinal fibrosis was scored as mild, moderate or severe depending on the density and extent of trichrome-positive connective tissue staining and disruption of tissue architecture^[38]. A quantitative estimate of mucosa, submucosa and muscular layer thickness of distal esophagus, proximal and distal small bowel, proximal and distal colon, and distal ureters was performed. Morphometric analysis was done in all animals by ten measurements randomly taken in 4 different fields (x 40) in a double blind fashion by two independent pathologists with an agreement always higher than 90%. Light microscopic and IHC microphotographs were taken by Olympus BX-51 Light Microscopy (Olympus, Optical Co. Ltd., Tokyo, Japan) with a videocam (Spot Insight, Diagnostic Instrument, Inc., Sterling Heights, MI, USA) and processed with an image analysis system (IAS-Delta Sistemi, Rome, Italy) software.

Immunohistochemistry

Samples from small bowel and colon obtained as previously described, were also promptly fixed with 10% buffered formalin in PBS (pH 7.4) for 3 h, dehydrated in graded ethanols and embedded in low-temperature-fusion paraffin. Serial 3 µm sections were incubated for 40 min in methanol and 3% hydrogen peroxide solution and then rinsed in PBS. Thereafter, sections were incubated overnight at 4 °C with monoclonal antibodies to CD3, TGFβ-1, Smad7 and α-SMA (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), used at a dilution of 1:100, 1:250, 1:100 and 1:400 respectively in PBS. Additional sections were incubated with a cocktail of monoclonal antibodies to collagen types (I, III, IV, V, VII) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) in order to demonstrate the morphological and topographic features of collagen distribution in different layers of small bowel and colonic wall.

Samples were then rinsed with PBS for 5 min and incubated with a labeled streptavidin-biotin-peroxidase conjugate kit (Dako LSAB, cod. K0675, Dako-Cytomation, Milan, Italy). After rinsed in PBS for 10 min, the sections were incubated with 3,3-diaminobenzidine-tetrahydrochloride (DAB, Sigma Aldrich, Milan, Italy) for 1-3 min.

To control specificity of the immune reaction, sections were incubated omitting the primary antibody, i.e., incubated with the secondary antibody alone. Finally, the samples were counterstained with Mayer's hematoxylin and observed under photomicroscope (Olympus BX51 light microscope; Olympus, Optical Co. Ltd, Tokyo, Japan).



Figure 2 Morphology of Smad3 null and wild-type mice. The majority of Smad3 null mice exhibited a reduced size compared to littermate controls. Severe bending of forepaw joints was present in Smad3 null mice.

Measurement of colonic TGF-β1 protein levels

TGF-β1 protein was determined by ELISA. Briefly, tissue was homogenized in the presence of a mixture of protease inhibitors with a broad specificity for the inhibition of serine, cysteine, aspartic proteases and aminopeptidases (1 mL/20 g). The mixture contained 4, (2-aminoethyl) benzenesulphonyl fluoride (AEBSF), pepstatin A, E-64, bestatin, leupeptin and aprotinin without metal chelators. For determination of TGF-β1 levels, an aliquot of the supernates was treated with 1 mol/L HCl to activate TGF-β1, followed by neutralization with 1 mol/L NaOH using a standard ELISA procedure (Quantikine, R&D Systems, Minneapolis, MN, USA).

Statistical analysis

All statistical analyses were performed in a double-blind fashion and results were computed using an appropriate program (SAS/STAT software). Results were expressed as mean±SD. Statistical significance was performed by the two-tailed Student's *t* test for paired data and *P*<0.05 was considered statistically significant.

RESULTS

Animal gross phenotype

The phenotype of Smad3 null mice at birth was identical to that in heterozygous and wild-type littermate controls. Nevertheless, the Smad3 null mice at three weeks of age were characterized by the following hallmarks: short body length compared to the tail and a predominant deformity of the anterior paws (Figure 2). About 20% of Smad3 null animals developed a wasting disease (Figure 3) and died between 3-6 mo but none of them developed diarrhea or hematochezia, and only a marked dilatation of the colon was observed in two Smad3 null mice.

About 80 % of null mice survived up to 6 mo of age, while a mortality rate of 6% and 2% was observed at 18 mo of age in heterozygous and wild-type mice, respectively. No serosal or mucosal macroscopic lesions of the small or large intestine were detected either in wild-type mice or in null mice.

Histologic and morphometric evaluation

HE staining of the small and large bowel showed a normal morphology both in wild-type mice and in Smad3 null mice (Figure 4). Masson trichromic staining and silver

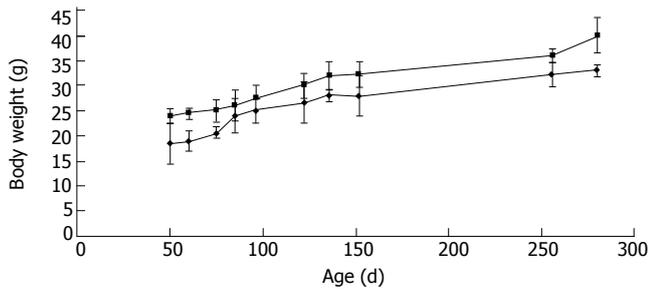


Figure 3 Body Weight changes of wild-type and Smad3 null mice. Each point represents mean weight data pooled from 10 mice. Standard deviations are indicated. Plot of weight (g) vs age (days). Wild-type are indicated as □ (squares), and null as ◇ (diamonds).

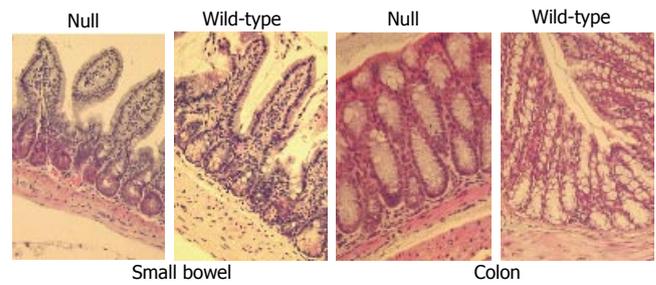


Figure 4 Haematoxylin and eosin-stained sections (x 20) analysis of small and large bowel from wild-type and Smad3 null mice shows normal morphology.

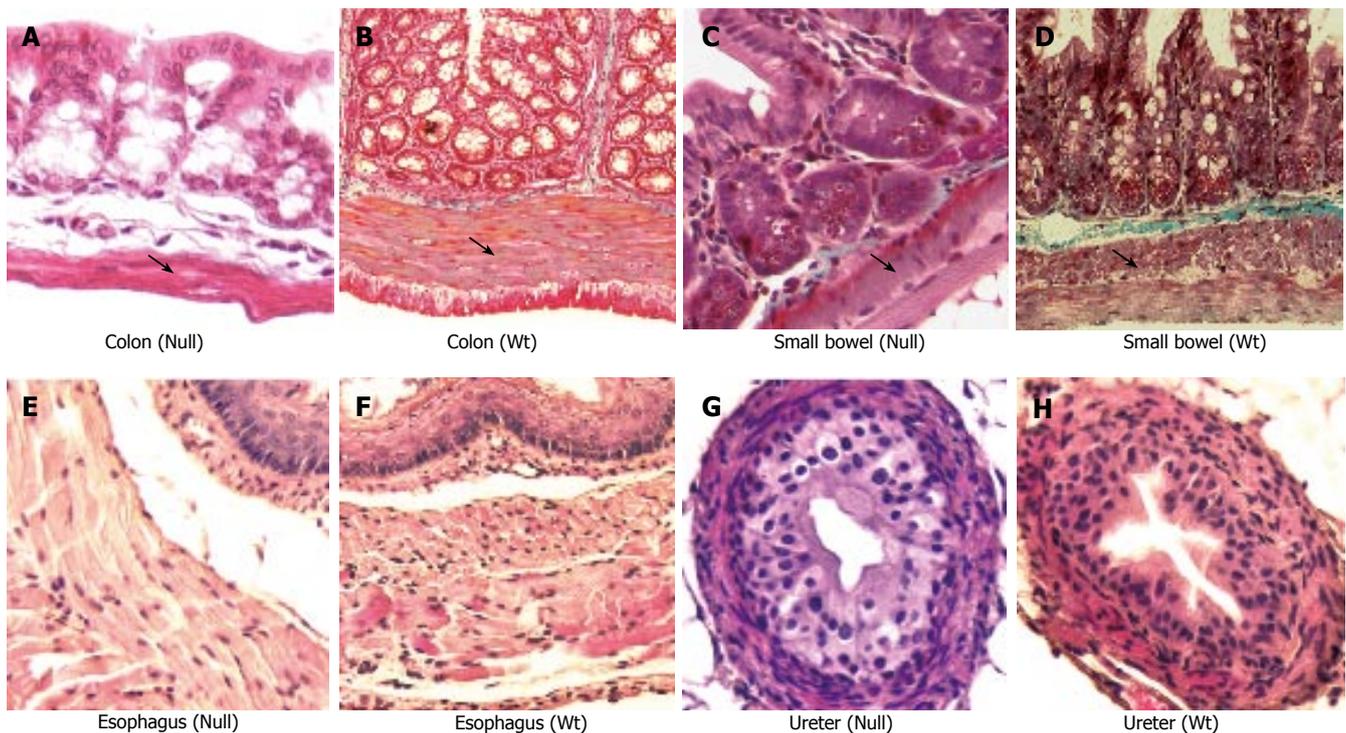


Figure 5 Masson trichrome staining (x 20) of small and large bowel from Smad3 mice. Significant reduction of muscular layer of descending colon of Smad3 null (A) is observed compared to colon from wild-type (WT) mice (B), reduction of muscle layer in cross sections of the proximal small bowel from Smad3 null (C) as compared to wild-type mice (D). Haematoxylin and eosin staining (x 20) of ureter and esophagus of Smad3 mice. Cross section of esophagus from Smad3 null (E) and wild-type mice (F) shows no differences in muscle layer. Cross section of ureter from Smad3 null (G) and wild-type mice (H) shows no differences in muscle layers.

staining of the colon and small intestine showed a similar collagen distribution in all intestinal layers both in wild-type mice and in Smad3 null mice (Figure 5).

A significant reduction in muscle layer thickness confined to the colon and small intestine was observed in Smad3 null mice compared to wild-type mice, while the mucosa and submucosa layers were similar in the two groups (Table 1, Figure 5). No differences in the thickness of the mucosa, submucosa and muscle layers of the ureter and esophagus were observed either in wild-type mice or in Smad3 null mice (Figure 5).

Immunohistochemistry evaluation

In the colonic and small intestine mucosa of Smad3 null mice, a significant staining of CD3+ T cells, TGF- β 1 and Smad7 was observed compared to the intestinal mucosa of wild-type mice (Figure 6). TGF- β 1 and Smad7 staining was localized mainly in lymphocytes of the intestinal lam-

ina propria from Smad3 null mice. In small intestine and colon, α -SMA staining was limited to the smooth muscle cells of muscularis mucosa, muscularis propria as well as myocytes of the median vessel layer, with a comparable pattern both in wild-type mice and in null mice (Figure 7). Staining of collagens I-VII of the small intestine and colon was localized mainly in the submucosa and muscularis propria connective tissue and did not differ between the wild-type and null mice (Figure 7).

TGF- β 1 levels in colon homogenates

TGF- β 1 levels in colonic tissue homogenates were significantly higher in null mice than in wild-type mice (Figure 8).

DISCUSSION

In this study, we characterized the changes in intestinal structures which may occur in a Smad3 knock out mouse

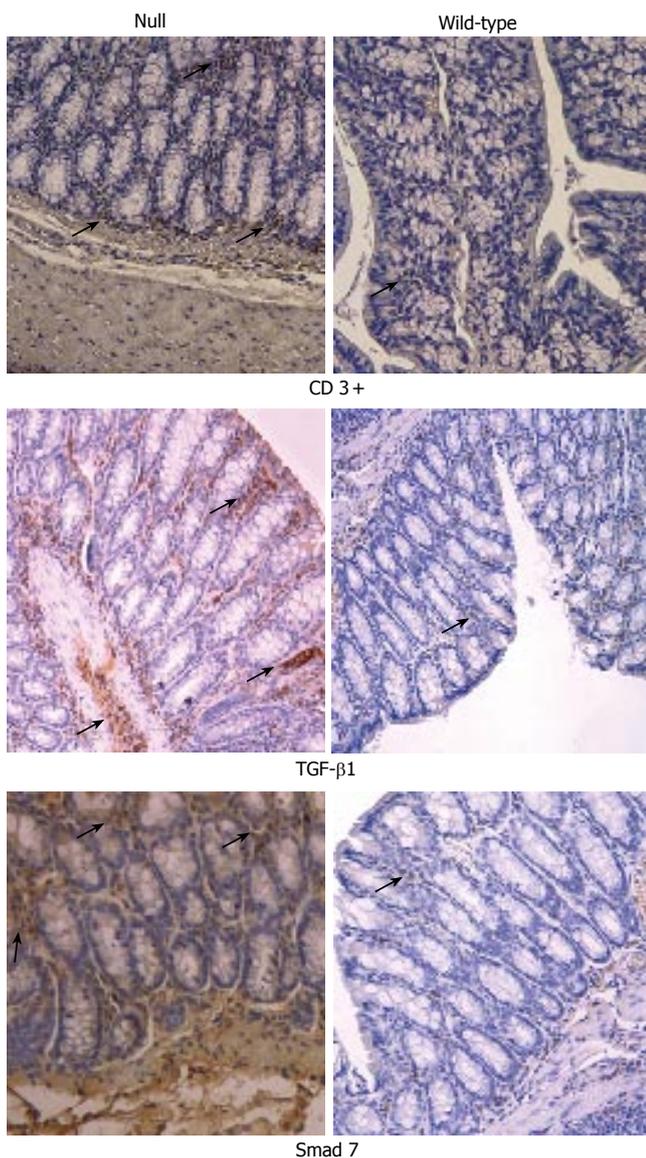


Figure 6 Immunohistochemical analysis (x 20) of CD3+ T cells, TGF-β1 and Smad7 in colon obtained from Smad3 null and wild-type mice. CD3+ T cells were significantly increased within large intestine of Smad3 null mice as compared to the wild-type mice. TGF-β1 and Smad7 were significantly increased within large intestine of Smad3 null mice compared to wild-type mice.

model compared to the littermate wild-type controls. In particular, attention was focused on evaluation of intestinal alterations present in healthy adult animals that could be used as a model to investigate the role of the TGFβ1/Smad3 pathway in the development of chronic intestinal inflammation and fibrosis. The animals studied did not develop any signs of colitis, but Smad3 null mice had a deficit in body weight gain as compared to their controls. Smad3 null mice were smaller than wild-type littermates and about 40% of them showed the presence of medially torqued forepaws associated with kyphosis sometimes. Some aspects of this phenotype are similar to those of mice expressing a transgenic dominant negative type II TGF-β receptor^[39]. We did not observe any significant macroscopic intestinal lesions except for colonic dilatation in 20% of Smad3 null mice.

Histopathological analysis of small and large intestine specimens did not reveal any neoplastic lesions, significant

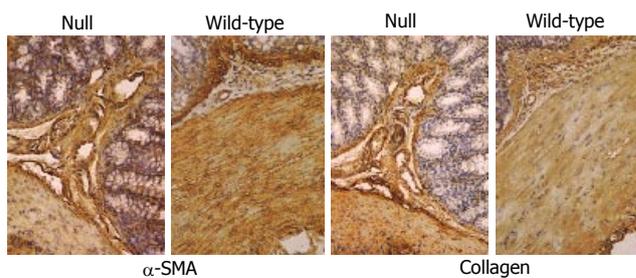


Figure 7 Immunohistochemical analysis (x 20) of α-SMA and collagens I-VII in colon from Smad3 null and wild-type mice. A similar localization of α-SMA antibody was found in miocytes of muscularis mucosae, muscle layer and vessels of both groups of animals. Staining of collagens I-VII in large intestine of Smad3 null and wild-type mice was localized mainly within connective tissue of submucosa and muscularis propria showing identical staining pattern between the two groups of mice.

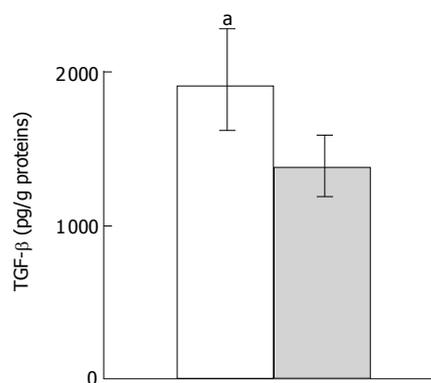


Figure 8 TGF-β1 ELISA of colon homogenates from Smad3 null (solid column) and wild-type mice (dashed column). Data are given as mean±SD. ^aP<0.05 vs wild type mice.

intestinal mucosa inflammation (i.e., chronic abscesses or marked neutrophil/monocyte infiltrate) or changes in intestinal connective tissue distribution. On the contrary, mice that died prematurely (1-3 mo of age) often showing signs of systemic inflammation, presented severe histologic lesions of the intestinal mucosa (data not shown) similar to the findings described earlier^[31]. These animals were not included in the present study since the aim of the investigation was to evaluate the intestine of healthy adult mice in which an intestinal fibrosis could be experimentally induced.

A significant reduction in smooth muscle layer thickness of small and large intestine was present in Smad3 null mice as compared to wild-type mice. These alterations of colonic smooth muscle layers could account for the colonic dilation observed in Smad3 null mice. In this respect, immunostaining of the colonic mioenteric plexus was also performed which showed a normal appearance (data not shown). Smooth muscle layer thickness from the esophagus and ureters was normal and similar in the two groups of mice. The reason why the intestinal muscle layer thickness was reduced in Smad3 null mice is unknown. Nevertheless, several lines of evidence suggest that TGF-β/Smad3 signalling plays an important role in the development of smooth muscle cells from totipotent or multipotent embryonic stem cells^[40,41]. Furthermore, TGF-β/Smad3 signalling is also involved in the differentia-

tion and proliferation of smooth muscle cells^[42,43].

A number of phenotypic changes as observed in our mice with a targeted disruption of Smad3 in exon 8^[31], are similarly present also in mice with a disruption of Smad3 in exon 1 or 2^[44,45]. In fact, in all these three models a decrease in size and growth rate, and the presence of skeletal abnormalities have been observed. They also show a decreased survival. The deletions of both exon 8 and 1 are associated with different degrees of intestinal inflammation^[31,44], while the deletion of exon 2 accompanies the development of metastatic colorectal cancer^[45]. The reason for this discrepancy is unknown. This discrepancy could be related to differences in genetic background of the Smad3 null animals used. It is possible that in mice a differential activation of downstream targets exists with a disruption in exons 1, 2 and 8. This hypothesis is supported by *in vitro* studies indicating that different domains of the Smad3 protein may be involved in activation of diverse downstream pathways^[46,47].

Although no evident intestinal mucosa inflammation was found, the immunohistochemical analysis showed an increase in CD3+ T cells within the intestinal mucosa of Smad3 null mice, compared to wild-type mice, which is consistent with previous data^[31]. In addition, increased TGF- β 1 and Smad7 staining was observed in the intestinal mucosa of Smad3 null mice. The constitutively high TGF- β expression in the intestine could account for the positive counteractive mechanism due to the loss of intracellular transduction signals in Smad3 null mice. TGF- β overexpression could not be attributed to monocytes and macrophages not increased in the intestinal mucosa^[31,32], nor to TGF- β autoinduction under the control of Smad3^[32,33]. On the other hand, increased TGF- β expression could be attributed to the increased T cells in the intestinal mucosa or to the platelet degranulation not assessed in this study. The mechanism that may upregulate Smad7 expression is not clear. Moreover, Smad7 is strongly and rapidly induced by TGF- β itself^[48]. Efficient expression of Smad7 appears to depend upon the cooperation of Smad, Sp1 and AP-1 transcription factors^[49]. In Smad3 null mice, the overexpression of TGF- β 1 may induce Smad2 phosphorylation which in turn could upregulate Smad7 intracellular expression even in the absence of Smad3. In some cell types, Smad7 expression is induced by other signalling pathways, such as Jak1/Stat1 pathway following stimulation with interferon (INF)- γ and activated nuclear factor (NF)- κ B following stimulation with tumor necrosis factor (TNF)- α ^[50,51]. Whatever the main inducible Smad7 factor is, high Smad7 expression levels interfere with activation of Smad2 and Smad3 or accelerate degradation of TGF- β receptors, inhibiting TGF- β /Smad signalling.

The lack of significant neutrophil/monocyte infiltrate in intestinal mucosa of Smad3 null mice could be related to the impaired chemotactic response toward TGF- β shown by mutant neutrophils and monocytes^[32,33]. In contrast, mutant-activated T cells are resistant to the suppressive effect of TGF- β leading to their significant increase in the intestinal mucosa^[31]. Furthermore, a reduced number of IgA+ plasma cells has been detected in the intestine of severely affected Smad3 null mice^[31]. These data suggest that Smad3 plays a pivotal role in TGF- β -mediated

regulation of mucosal immunity and local inflammatory response. Loss of these functions may also be responsible for infection and the high mortality rate of Smad3-null mice.

It has been reported that mice lacking Smad3 show accelerated healing of cutaneous incisional wounds with reduced inflammation and accumulation of matrix^[32,33], and decreased cutaneous lesions and fibrosis after exposure to ionizing radiation^[34] or subcutaneous injection of bleomycin^[52]. Furthermore, loss of Smad3 could attenuate bleomycin-induced lung fibrosis^[53], CCl4-induced liver fibrosis^[54], and glomerular fibrosis induced by different methods^[55] in mice, suggesting that Smad3 plays a pivotal role during tissue injury that leads to skin, lung, liver and kidney fibrosis^[56].

Recently, it has also been reported that reduction of Smad3 accelerates re-epithelization in a murine model of colitis^[57]. Amelioration of cutaneous radiation-induced fibrosis has also been obtained by halofuginone, which inhibits the formation of phospho-Smad2 and Smad3, increases inhibitory Smad7 expression, and decreases cytosolic and membrane TGF- β type II receptors^[58]. There is increasing evidence that Smad3 may take part in recruitment of fibroblasts to the site of injury, differentiation of fibroblasts to myofibroblasts and regulation of collagen synthesis^[33,34,59]. Since loss of Smad3 interferes with the effects of TGF- β on chemotaxis and autoinduction in inflammatory cells, and as well as induction of many ECM genes by TGF- β is also Smad3-dependent, this may explain why Smad3 null mice are resistant to cutaneous^[33,34,52], pulmonary^[53], hepatic^[54] and renal fibrosis^[55]. Based on these observations, we may hypothesize that mice lacking Smad3 would be also resistant to chronic intestinal inflammation and fibrosis. In fact, by inducing intestinal fibrosis with TNBS according to the protocol of Lawrance *et al*^[38], we observed a significant reduction of intestinal fibrosis in knock out mice as compared to the wild-type mice in preliminary experiments (data not shown).

In conclusion, about 80% of Smad3 knock-out mice survive up to 6 mo of age without developing significant macroscopic and histological lesions of the small and large intestine, with the exception of a reduction in intestinal muscle layer thickness. Preliminary data shown that Smad3 knockout mice are resistant to intestinal fibrosis. This model could be a useful tool to unravel the molecular mechanisms of chronic intestinal inflammation and fibrosis.

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Neutrophil depletion-but not prevention of Kupffer cell activation-decreases the severity of cerulein-induced acute pancreatitis

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Abstract

AIM: To determine whether neutrophil depletion and Kupffer cell inhibition might combine their protective effects to decrease the severity of acute pancreatitis.

METHODS: Mice had cerulein administration to induce acute pancreatitis and were pretreated with either anti-mouse neutrophil serum or gadolinium chloride (GdCl₃) to prevent Kupffer cell activation, or both treatments. Injury was assessed in pancreas and lungs. Myeloperoxidases (MPO) assessed neutrophil infiltration. Interleukin-6 (IL-6) and IL-10 were measured in serum, pancreas, lungs and liver.

RESULTS: In mice with acute pancreatitis, neutrophil depletion reduced the severity of pancreatitis and pancreatitis-associated lung injury. Kupffer cell inactivation by GdCl₃ had less protective effect, although IL-6 and IL-10 concentrations were significantly decreased. The protective treatment brought by neutrophil depletion was not enhanced by Kupffer cell

inactivation and both treatments did not combine their protective effects.

CONCLUSION: Our results confirm the role of activated neutrophils in aggravating organ injury in acute pancreatitis while the role of Kupffer cell activation is less obvious.

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Key words: Acute pancreatitis; Cytokines; Neutrophils; Kupffer cells; Pulmonary injury

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INTRODUCTION

During acute pancreatitis, the pathophysiology of pancreatic injury includes inflammatory processes and, following early activation of trypsinogen in acinar cells, recruitment of inflammatory cells aggravates pancreatic damage^[1,2]. Upon activation, circulating inflammatory cells adhere to vascular endothelial cells and transmigrate across the endothelial barrier within injured areas in pancreas as well as in remote organs. Thus, mice deficient in adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1)^[3] on endothelial cells or animals treated with an antineutrophil serum (ANS)^[3,4] are protected from pancreatitis and remote injuries. However, the protection remained incomplete^[3,4].

In contrast to the severe lung injury frequently associated with pancreatitis, hepatic injury is minor during acute pancreatitis^[5]. Nevertheless, hepatic functions are modified by pancreatitis. Detoxification is altered early in the evolution of the disease^[6,7] and this decreased hepatic detoxification is compensated by an increased detoxification in blood, lungs, and intestine macrophages^[7]. Moreover, mediators released from the damaged pancreas directly activate Kupffer cells and promote important inflammatory responses in the liver.

Elastase administration, either in the peritoneum^[8] or in perfused livers^[9] mimics this important inflammatory response. The role of the liver in acute pancreatitis is then to propagate pancreatic injury to lungs. Portocaval shunts (that dilute harmful pancreatic mediators in the systemic circulation)^[5,10] and inhibitors of Kupffer cell activation such as gadolinium chloride (GdCl₃)^[11] and liposome-encapsulated dichloromethylene diphosphonate (DMDP)^[12] decrease acute pancreatitis-associated-lung injury.

Because neutrophil depletion or prevention of Kupffer cell activation is only partially effective in decreasing the severity of acute pancreatitis, the aim of our study was to determine whether both treatments might combine their protective effects.

MATERIALS AND METHODS

Animals

Breeding pairs of C57BL6 mice were purchased from Charles River (Saint-Germain sur l'Arbreles, France) and bred and housed in temperature-controlled (23 ± 2 °C) cages with a 12 h light/dark cycle. They were fed with standard laboratory chow, given water *ad libitum*, and randomly assigned to control or experimental groups. The experimental protocol was reviewed and approved by the Ethics Committee for Animal Research of the University of Geneva and the Veterinary Office in Geneva and followed the guidelines for care and use of laboratory animals.

Induction of acute pancreatitis

Male mice (20-22 g) were intraperitoneally injected hourly for 10 h (10 injections) with a supramaximally stimulating dose of cerulein (CER, 50 µg/kg in 0.2 mL saline solution) to elicit a secretagogue-induced pancreatitis. Cerulein, the analog of the pancreatic secretagogue cholecystokinin was purchased from Research Plus (Bayonne, NJ, USA). Control (CONT) mice were injected with saline solution. One hour after the final injection, mice were sacrificed by a lethal intraperitoneal injection of pentobarbital.

Neutrophil depletion

Twelve hour before the start of cerulein administration, mice were treated with 0.2 mL rabbit anti-mouse neutrophil serum (ANS, ip injection, Accurate Chemical and Scientific Corp, Westbury, NY) as previously described^[3].

Kupffer cell inactivation

To prevent Kupffer cell activation, gadolinium chloride (GdCl₃, 1 mg/100 g of body weight, Sigma, Basel, Switzerland) was injected into mice tail vein 12 h and 1 h before the start of cerulein or saline administration. Another experimental group received both treatments.

Experimental groups

Five experimental groups were studied: CONT mice had saline injection and no treatment; CER mice had cerulein administration and were treated with saline; CER + ANS mice had cerulein administration and were treated with

anti-mouse neutrophil serum; CER + GdCl₃ mice had cerulein administration and were treated with gadolinium chloride; CER + ANS + GdCl₃ mice had cerulein administration and were treated with ANS and GdCl₃.

Quantification of cerulein-induced injuries

At the time of sacrifice (1 h following the last cerulein injection), blood was collected from the heart, centrifuged for 10 min, and the serum was kept at -80 °C until assayed. Serum amylase activity was measured using 4,6-ethylidene (G1)-p-nitrophenyl (G1)-αD-malto-heptoside (Sigma Chemical Company, St. Louis, Missouri) as substrate.

To quantify pancreatic edema, part of the pancreas was removed to measure tissue water content. Pancreatic tissue was weighed before and after desiccation at 95 °C during 24 h. The difference between the wet and dry tissue weights was calculated and expressed as a percent of tissue wet weight. The other parts of pancreatic tissues were frozen in liquid nitrogen for cytokine measurements.

Neutrophil sequestration within pancreas was detected by measuring tissue myeloperoxidase activity (MPO)^[13]. Pancreatic samples were thawed, homogenized in 1 mmol/L in 20 mmol/L phosphate buffer (pH 7.4), centrifuged (10000 g, 10 min), and the resulting pellet was resuspended in 50 mmol/L phosphate buffer (pH 6.0) containing 0.5% hexadecylmethylammonium bromide. The suspensions were subjected to four cycles of freezing and thawing and sonicated. The samples were centrifuged (10000 g, 5 min) and the supernatants were used to measure MPO activity. The reaction solution included 1.6 mmol/L tetramethylbenzidine (Sigma, Switzerland), 80 mmol/L sodium phosphate buffer (pH 5.4) and 0.3 mmol/L hydrogen peroxide (Sigma, Switzerland). Samples in solution were incubated at 37 °C for 2 min and the absorbance was read by an autoanalyzer. MPO activity was expressed as U/mg tissue dry weight.

Pulmonary microvascular permeability was evaluated by quantitating the leakage of intravenously injected fluorescein isothiocyanate (FITC)-labeled bovine albumin (0.5 mg/kg in 0.2 mL) into the bronchoalveolar space (BAL)^[14]. Immediately after sacrifice, the trachea was exposed and the lungs were injected three times with saline solution (1 mL). The lavage fluid was collected and FITC fluorescence was measured in the lavage fluid and serum using a Hitachi fluorospectrophotometer (excitation wave: 494 nm; emission wave: 520 nm). The ratio of fluorescence between BAL and blood was calculated and expressed the pulmonary permeability index.

Measurements of cytokines

Interleukin-6 (IL-6) and interleukin-10 (IL-10) were quantified in serum, pancreas, lung and liver using the commercially available enzyme linked immunosorbent assay kits (R&D, Abingdon, UK). Freshly isolated tissues were homogenized in 1.5 mL phosphate buffer (20 mmol/L, pH 7.4). After centrifugation (14000 g for 5 min at 4 °C), IL-6 and IL-10 concentrations were measured in the supernatant according to the manufacturer's recommendations and expressed as pg per mg proteins in tissues and pg/mL in serum. The mean concentrations of IL-6 and IL-10 mea-

Table 1 Pancreatic injury in control (CONT) mice and mice treated with cerulein (CER)

| | CONT | CER | |
|--|--------------------|---------------------|--------------|
| Serum amylase (UI/L) | 1582 [1236 - 1850] | 19528 [16369-26457] | $P < 0.0001$ |
| Pancreatic water content (% wet weight) | 0.74 [0.71 - 0.76] | 0.80 [0.78 - 0.83] | $P < 0.0001$ |
| Pancreatic MPO activity (U/mg tissue dry weight) | 322 [125 - 456] | 2738 [1978 - 3245] | $P = 0.0002$ |
| Pancreatic necrosis (%) | 1.26 [0.5 - 2.3] | 27.7 [24.5 - 35.0] | $P = 0.0001$ |

MPO=myeloperoxidase. $n \geq 5$ in each group. Data in CER and CONT groups were compared with Mann-Whitney test.

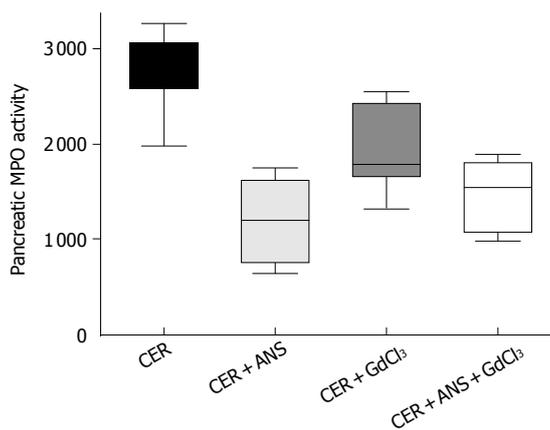


Figure 1 Pancreatic myeloperoxidase (MPO, U/mL tissue dry weight) activity in mice injected with cerulein and saline (CER), cerulein and antineutrophil serum (CER+ANS), cerulein and gadolinium chloride (CER+GdCl₃), CER+ANS+GdCl₃. $n \geq 5$ in each group. Kruskal Wallis analysis with Dunn posthoc test found a significant difference between CER and CER+ANS or CER+ANS+GdCl₃ mice.

sured in CER mice served as the 100% baseline values.

Morphology

At the time of sacrifice, pancreatic tissues were rapidly removed, fixed in 4% neutral phosphate-buffered formalin, embedded in paraffin, and sectioned (5 μm). After staining with hematoxylin-eosin, sections were examined by two morphologists who were not aware of the sample identity. The extent of acinar cell necrosis was quantified by computer-assisted morphometry and expressed as a function of total acinar tissue. Acinar cell necrosis included destruction of normal pancreatic architecture in combination with inflammation. After exclusion of non-acinar cells (islets of Langerhans and perivascular and periductular adventitial tissue), the amount of acinar necrosis were morphometrically quantified using a computerized image analysis video unit (Zeiss Camera, Zeiss, Bern, Switzerland).

For pulmonary morphology, a polyvinyl catheter was inserted into the trachea and used to instill 4% neutral buffered formalin into the lungs with a hydrostatic pressure of 30 cm H₂O. Formalin-distended lungs were harvested, fixed, paraffin-embedded, sectioned (5 μm) and stained.

Statistical analysis

Data are median [minimum - maximum] and differences

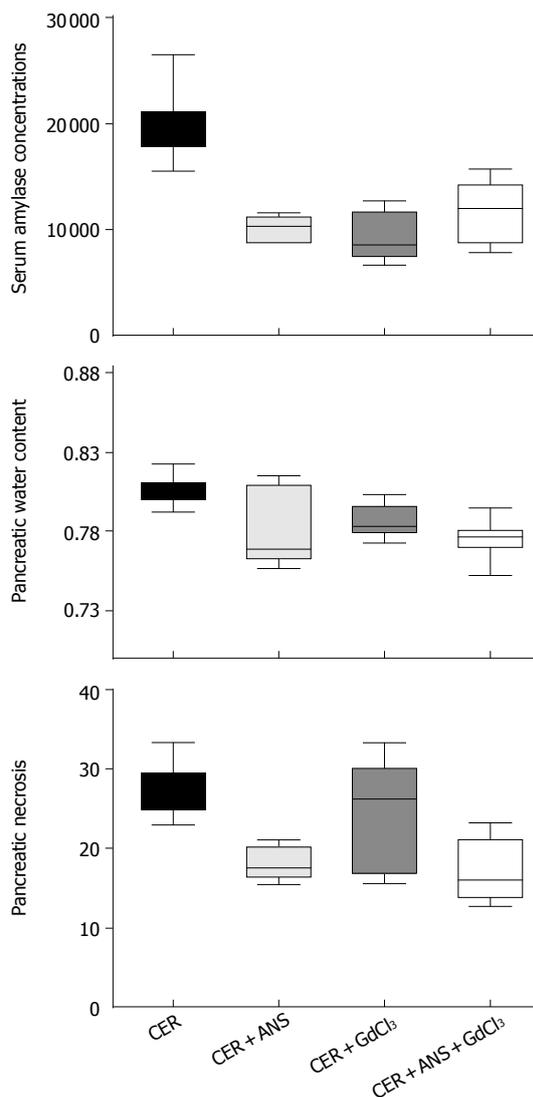


Figure 2 Serum amylase concentration (UI/L), pancreatic water content (% wet weight), and pancreatic necrosis (%) in mice injected with cerulein and saline (CER), cerulein and antineutrophil serum (CER+ANS), cerulein and gadolinium chloride (CER+GdCl₃), CER+ANS+GdCl₃. $n \geq 5$ in each group. Kruskal Wallis analysis with Dunn posthoc test found a significant difference between CER and CER+ANS, CER+GdCl₃, and CER+ANS+GdCl₃ (amylase concentrations) and CER and CER+ANS or CER+ANS+GdCl₃ mice (pancreatic water content and pancreatic necrosis).

between groups were analyzed by Mann-Whitney test (comparison between CONT and CER mice) or Kruskal-Wallis and Dunn posthoc tests (comparisons between CER, CER+ANS, CER+GdCl₃, and CER+ANS+GdCl₃). A P value < 0.05 was considered significant.

RESULTS

Acute pancreatitis

Cerulein administration induced severe acute pancreatitis. Serum amylase concentrations and pancreatic water content markedly increased in CER mice in comparison to CONT mice (Table 1). Pancreatic injury was also characterized by an increase in acinar cell necrosis and MPO activity.

Pre-treatment with ANS before acute pancreatitis induction significantly decreased pancreatic MPO activity ($P < 0.001$, Figure 1) with a concomitant decrease in

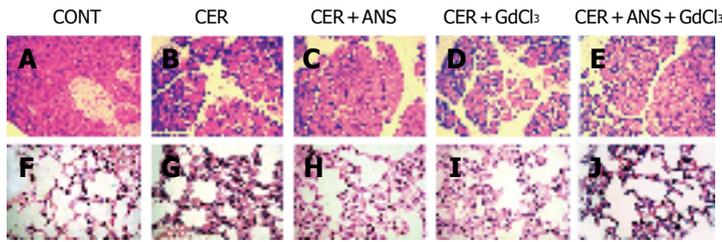


Figure 3 Representative sections of pancreas (upper panels) and lung (lower panels) stained with hematoxylin and eosin. Mice were injected with saline solution (CONT, A and F), cerulein (CER, B to E and G to J) and treated with antineutrophil serum (CER + ANS, C and H), gadolinium chloride (CER + GdCl₃, D and I) or both treatments (CER + ANS + GdCl₃, E and J). These illustrations represent sections from ≥ 3 mice. Magnification x400.

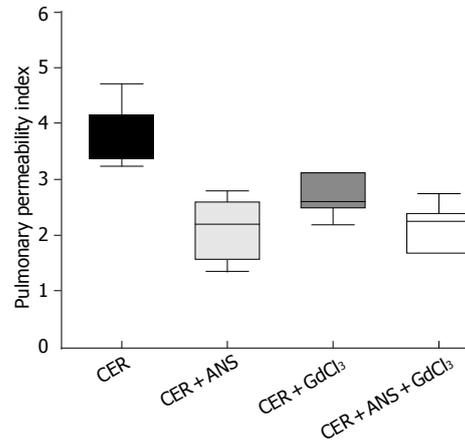
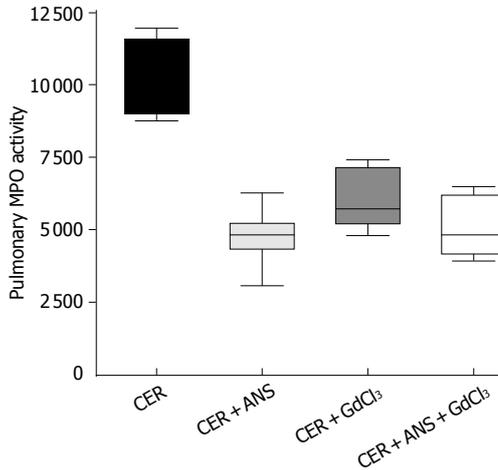


Figure 4 Pulmonary myeloperoxidase activity (MPO, U/mg tissue dry weight) and pulmonary permeability index in mice injected with cerulein and saline (CER), cerulein and antineutrophil serum (CER + ANS), cerulein and gadolinium chloride (CER + GdCl₃), CER + ANS + GdCl₃. *n* ≥ 5 in each group. Kruskal Wallis analysis with Dunn posthoc test found a significant difference between CER and CER + ANS or CER + ANS + GdCl₃ mice (pulmonary MPO and permeability index).

Table 2 Pulmonary injury in control (CONT) mice and mice treated with cerulein (CER)

| | CONT | CER | |
|---|------------------|----------------------|-------------------|
| Pulmonary MPO activity (U/mg tissue dry weight) | 947 [782 - 1145] | 10173 [8710 - 12035] | <i>P</i> < 0.0001 |
| Pulmonary permeability index | 0.57 [0.5 - 0.8] | 3.9 [3.1 - 5.1] | <i>P</i> = 0.0002 |

MPO = myeloperoxidase. Pulmonary permeability index: ratio of FITC-labeled bovine albumin between bronchoalveolar lavage and serum. *n* ≥ 5 in each group. Pulmonary MPO activity and pulmonary permeability index were compared by Mann-Whitney test.

pancreatic injury: serum amylase concentration (*P* < 0.01, Figure 2), pancreatic water content (*P* < 0.01, Figure 2), and acinar cell necrosis significantly decreased (*P* < 0.01, Figure 2). However, pre-treatment with ANS did not fully protect against pancreatic injury. Morphologic studies confirmed these findings (Figure 3).

Prevention of Kupffer cells activation by GdCl₃ decreased serum amylase concentration (*P* < 0.05) but did not change significantly pancreatic necrosis and pancreatic water content (Figure 2). GdCl₃ had no effect on pancreatic MPO activity (Figure 1). The decreased pancreatic injury was similar CER + ANS and CER + ANS + GdCl₃ mice.

Acute pancreatitis-associated lung injury

Because acute pancreatitis is frequently associated with injury in remote organs, we determined the consequences of both treatments on lung injury. CER administration increased pulmonary MPO activity and the pulmonary permeability index (Table 2). Lung injury is also evidenced by a marked thickening of the alveolar-capillary membrane (Figure 3).

Table 3 IL-6 and IL-10 concentrations in serum, pancreas, lungs, and livers in control (CONT) mice and mice treated with cerulein (CER)

| | CONT | CER | |
|----------------------------------|--------------------|----------------------|------------------|
| Serum IL-6 (pg/mL) | 8.9 [8.7 - 11.4] | 267.5 [98.0 - 312.0] | <i>P</i> = 0.02 |
| Serum IL-10 (pg/mL) | 11.8 [8.9 - 14.7] | 18.7 [10.9 - 21.6] | <i>P</i> = 0.23 |
| Pancreatic IL-6 (pg/mg protein) | 1.2 [0.5 - 1.9] | 8.4 [6.4 - 13.2] | <i>P</i> = 0.02 |
| Pancreatic IL-10 (pg/mg protein) | 0.08 [0.03 - 0.20] | 0.32 [0.20 - 0.79] | <i>P</i> = 0.03 |
| Pulmonary IL-6 (pg/mg protein) | 1.5 [0.5 - 2.1] | 3.0 [2.4 - 6.9] | <i>P</i> = 0.004 |
| Pulmonary IL-10 (pg/mg protein) | 0.10 [0.01 - 0.20] | 0.87 [0.42 - 3.80] | <i>P</i> = 0.06 |
| Hepatic IL-6 (pg/mg protein) | 1.15 [0.80 - 1.60] | 8.20 [5.60 - 11.52] | <i>P</i> = 0.006 |
| Hepatic IL-10 (pg/mg protein) | 0.12 [0.05 - 0.20] | 0.43 [0.33 - 0.48] | <i>P</i> = 0.06 |

n ≥ 5 in each group. Data in CER and CONT groups were compared by Mann-Whitney test.

Pre-treatment with ANS significantly decreased MPO activity (*P* < 0.001) and the pulmonary permeability (*P* < 0.01) induced by CER, while GdCl₃ did not (Figure 4). Pulmonary injury was similarly prevented in CER + ANS and CER + ANS + GdCl₃ mice.

Cytokine expression

CER administration greatly increased IL-6 concentrations in serum but not that of IL-10 (Table 3). CER administration increased expression of both cytokines in liver, pancreas, and lungs.

GdCl₃ pretreatment significantly attenuated IL-6 and IL-10 concentrations in liver (*P* < 0.01 for both IL),

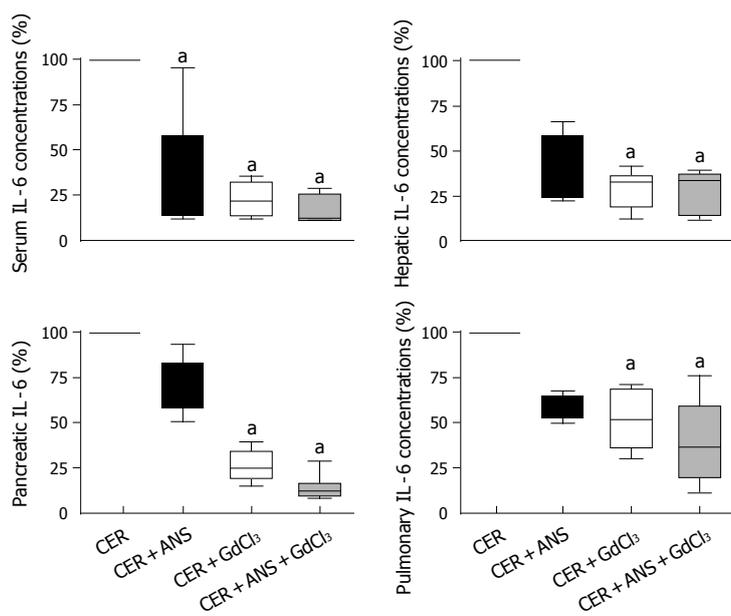


Figure 5 Interleukin-6 (IL-6, %) concentrations in serum, pancreas, lung and liver the 100% value corresponding to the mean value measured in mice injected with cerulein (CER). Mice were injected with cerulein and saline (CER), cerulein and antineutrophil serum (CER+ANS), cerulein and gadolinium chloride (CER+GdCl₃), and CER+ANS+GdCl₃. $n \geq 5$ in each group. ^a $P < 0.05$ vs CER.

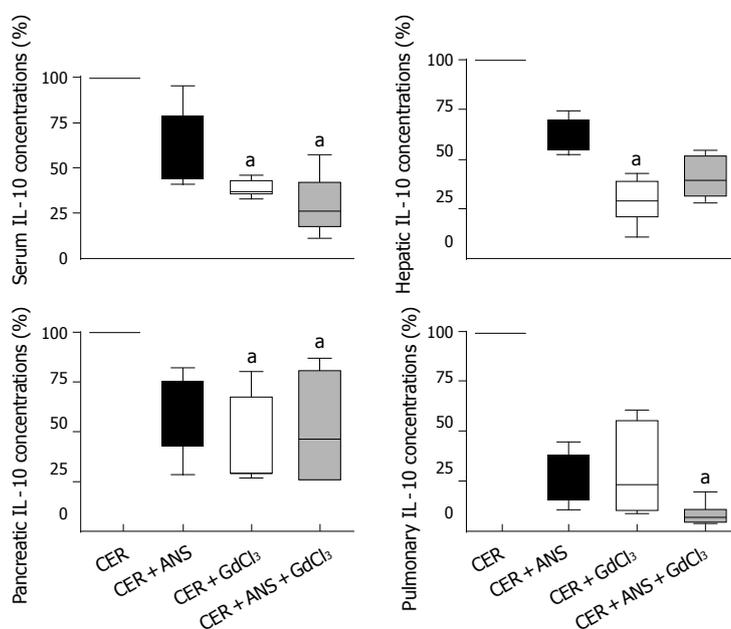


Figure 6 Interleukin-10 (IL-10, %) concentrations in serum, pancreas, lung and liver the 100% value corresponding to the mean value measured in mice injected with cerulein (CER). Mice were injected with cerulein and saline (CER), cerulein and antineutrophil serum (CER+ANS), cerulein and gadolinium chloride (CER+GdCl₃), and CER+ANS+GdCl₃. $n \geq 5$ in each group. ^a $P < 0.05$ vs CER.

pancreas ($P < 0.05$ and $P < 0.01$ respectively), and serum ($P < 0.05$ for both IL-6 and IL-10) (Figures 5 and 6). IL-6 concentrations in lungs were decreased by GdCl₃ ($P < 0.05$) but IL-10 concentrations were not. ANS did not significantly modify IL-6 and IL-10 concentrations in liver, pancreas, and lungs. IL-6 concentrations in serum was decreased ($P < 0.05$) but serum IL-10 concentrations were not modified by ANS pretreatment.

DISCUSSION

In mice with acute pancreatitis, neutrophil depletion reduced the severity of pancreatitis and pancreatitis-associated lung injury. Kupffer cell inactivation by GdCl₃ had less protective effect, although IL-6 and IL-10 concentrations in tissues were significantly decreased. The protective treatment brought by neutrophil depletion was not enhanced by inhibition of Kupffer cell activation and both treatments did not combine their protective effects.

Neutrophil infiltration in pancreas and lungs

Following cerulein injections in mice, neutrophil infiltration (assessed by MPO activity) was observed 12 h after the first injection. However, we previously showed that MPO activity peaks later by 24 h in pancreas and 36 h in lungs after the first injection and remains elevated by d 7^[14]. This neutrophil infiltration in tissues plays an important role in the development of acute pancreatitis and pancreatitis-associated lung injury. Thus, mice deficient in adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) on endothelial cells^[3] or animals treated with an antineutrophil antibody^[3,4] are protected from pancreatitis and remote injuries. However, the protection remained partial^[3,4]. This incomplete protection suggests that either the dose of ANS used is insufficient to completely remove all neutrophils or that additional pathways must be targeted to obtain a full protection. We previously showed that ANS injection (0.2 mL) in mice reduced the circulating neutrophils by 85%^[3] while 0.5 mL/100 g in rats decreased

the circulating neutrophils to below 200 cells/mL^[4]. Thus, neutrophil depletion with ANS is correct and the residual MPO activities in lungs and pancreas after ANS treatment (Figures 2 and 4) might reflect the presence of local macrophages. An incomplete protection by ANS has already been shown in mice^[3] or rats^[4] treated with cerulein. Consequently, an additional independent pathway, Kupffer cell inactivation, was targeted to further prevent acute pancreatitis.

Role of the liver in acute pancreatitis

The role of the liver in propagating pancreatic injury to lungs has been evidenced by inactivating Kupffer cell or diverting blood from the pancreatic vein to the systemic circulation. Thus, portocaval shunts (that dilute harmful pancreatic mediators in the systemic circulation)^[5,10] and inhibitors of Kupffer cell activation such as gadolinium chloride (GdCl₃)^[11] and liposome-encapsulated dichloromethylene diphosphonate (DMDP)^[12] significantly decrease acute pancreatitis-associated-lung injury. As expected, we showed that GdCl₃ pretreatment decreased IL-6 and IL-10 concentrations in the liver (Figures 5 and 6). In isolated rat livers, perfusion of pancreatic elastase activates Kupffer cells with subsequent NF- κ B activation and TNF- α overproduction and this effect is abolished by GdCl₃ pre-treatment^[9]. GdCl₃ is specifically taken up by Kupffer cells and is not toxic by itself (when the injected dose is 100 mg/kg, iv), as previously published by Gloor *et al*^[15]. However, in our study, the pulmonary permeability index and MPO activity were not significantly decreased in comparison to CER mice (Figure 4). Pulmonary IL-6 concentrations in CER + GdCl₃ mice were lower than in CER mice but the IL-10 concentration decrease did not reach significance.

GdCl₃ pretreatment also decreased serum IL-6 and IL-10 concentration as previously published^[15]. In contrast to Gloor *et al*^[15] we found that GdCl₃ also decreased pancreatic IL-6 and IL-10 concentrations, as well as serum amylase concentration. Pancreatic necrosis, water content, and pancreatic MPO were not different between CER + GdCl₃ mice and CER mice, as previously described^[15]. Our results either question the specific inactivation of hepatic macrophages by GdCl₃ or suggest that the decreased cytokine concentrations in pancreas reflect a decreased systemic inflammatory response. In a severe form of acute pancreatitis, GdCl₃ pretreatment was able to reduce the mortality rate^[15]. The beneficial effect of GdCl₃ on the mortality rate remains puzzling because GdCl₃ administration decreases IL-10 concentrations, a cytokine which has a protective effect on the evolution of acute pancreatitis^[16,17].

Kupffer cell inactivation has also been induced by other drugs such as liposome-encapsulated dichloromethylene diphosphonate which decreases apoptosis and TGF- β production in liver^[12] or CNI-1493 which reduced pancreatitis-associated liver injury through TNF- α and IL-1 expression^[18].

In conclusion, our results confirm the role of activated neutrophils in aggravating organ injury in acute pancreatitis while the role of Kupffer cell activation is less obvious. Another more early event should probably be targeted in

combination with neutrophil depletion to fully prevent the initiation of the disease.

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Effect of hypercholesterolemia on experimental colonic anastomotic wound healing in rats

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Abstract

AIM: To evaluate the mechanical and biochemical parameters of colonic anastomotic healing in hypercholesterolemic rats.

METHODS: Sixty rats were divided into two groups of 30 each according to their dietary regimens. The test group was fed with a high cholesterol-containing diet for two months while the control group had standard diet. These two groups were further divided into three subgroups consisting of ten rats each. After hypercholesterolemia was established, left colon resection and anastomosis were performed in both groups and samples from liver and abdominal aorta were taken to evaluate the systemic effects of hypercholesterolemia. Anastomotic wound healing, blow-out pressures and tissue hydroxyproline levels were evaluated.

RESULTS: The test group had a significant weight gain in two months. Microscopic examination of the abdominal aorta revealed no atherosclerotic change in none of the groups, but liver tissue specimens showed significant steatosis in the test group. Tissue hydroxyproline levels and anastomotic blow-out pressures were significantly lower in the test group than in the controls.

CONCLUSION: Hypercholesterolemia not only increases hydroxyproline levels and blow-out pressures but also worsens anastomotic wound healing.

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Key words: Hypercholesterolemia; Colonic anastomosis; Anastomotic wound healing

INTRODUCTION

Hypercholesterolemia is the major etiologic factor for atherosclerosis in Western countries^[1]. However, the effects of hypercholesterolemia on anastomotic wound healing have not been studied.

Leakage in colonic anastomosis has a higher incidence of morbidity and mortality rate compared with that in small intestinal anastomosis. The overall leakage incidence of intestinal anastomosis is 2-35%^[2]. Most of them are minimal and can be limited by the host defense mechanisms. Systemic and local factors play a role in anastomotic wound healing. Anemia, hypovolemia, low arterial PO₂, neutropenia, low O₂ saturation, malnutrition, vitamin deficiencies, zinc deficiency, jaundice, uremia and high dose corticosteroids are some of the systemic factors, while local factors include infection, intestinal contents, prophylactic antibiotics, technique of suturing and suture material, radiation and mesenteric vascular occlusion^[2-4].

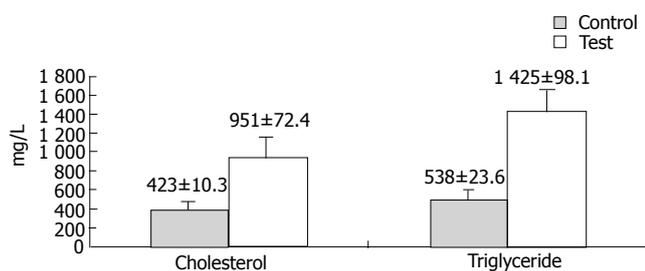
In experimental studies, microscopic healing in small intestine is better than that in the colon^[5,6]. Intestinal anastomotic wound healing can be evaluated mechanically, biochemically and histopathologically. Mechanical evaluation depends on the blow-out pressure of the anastomotic line. This pressure can change with the inflation rate of the intestine or the in situ procedure^[2,7-9]. Continuous suturing technique has no effect on this pressure^[10].

Biochemically, collagen production rate and amount in the anastomosis line are the factors to be measured. Since the majority of the collagens are hydroxyproline, all measurements are made by detecting the hydroxyproline content^[7,11,12]. Histopathologically, the only evidence is the infiltration of several kinds of inflammatory cells^[2,7].

In this study, the mechanical and biochemical parameters of colonic anastomotic wound healing were evaluated in rats fed with a high cholesterol containing diet (2% cholesterol + 1% colic acid: Sigma Co., Manchester, UK) for two months^[13]. Abdominal aortic and liver tissue samples were also obtained to evaluate the systemic effects of hypercholesterolemia.

Table 1 Pre- and post-feeding body weights of hypercholesterolemic rats

| | Pre-feeding weight (g) | Post-feeding weight (g) |
|--------|------------------------|-------------------------|
| Rat 1 | 250 | 325 |
| Rat 2 | 255 | 330 |
| Rat 3 | 245 | 350 |
| Rat 4 | 240 | 310 |
| Rat 5 | 260 | 325 |
| Rat 6 | 250 | 335 |
| Rat 7 | 275 | 360 |
| Rat 8 | 280 | 355 |
| Rat 9 | 270 | 350 |
| Rat 10 | 240 | 340 |
| Mean | 256.5 | 338 |

**Figure 1** Mean cholesterol and triglyceride level in test and control groups.

MATERIALS AND METHODS

Experimental animals and grouping

Sixty rats of both sexes weighing 240-300 g used in this study were divided into two groups according to their dietary regimens. The test group consisted of 30 rats fed with a high cholesterol diet for two months while the control group had standard diet for the same period. The test group had a significant weight gain at the end of this period (Table 1).

These two groups were further divided into three subgroups, ten rats each group. Ten milliliter blood was taken from each rat for measurement of serum total cholesterol and triglyceride levels. In the test group, serum total cholesterol and triglyceride levels were significantly higher than in the control group (Figure 1). After hypercholesterolemia was established, left colon resection and anastomosis were performed in both groups and tissue samples were taken from liver and abdominal aorta.

Surgical technique

After an overnight fasting, intramuscular ketamin HCl (Ketalar, Parke Davies) was administered (50 mg/kg). One percent povidon-iodine was used for local cleansing. A midline laparotomy was performed and a segment of left colon (1 cm) was resected. Colo-colonic anastomosis was made using 7/0 polypropylene (Prolene, Ethicon Inc., UK) with 8-10 stitches for a layer. There was no postoperative mortality.

**Figure 2** Insertion of an infusion pump into the colon.**Figure 3** Anastomosis line.

In both groups, 10 rats underwent relaparotomy on the third day and 10 rats on the seventh day. Colon lumen was obliterated 10 cm distal to the anastomosis using 3/0 silk ligature. Colon was transected 10 cm proximal to the anastomotic line and an infusion set was inserted into the lumen. This set was connected to an infusion pump (Life Care 5000 Infusion System, Abbot, Illinois, USA) and isotonic sodium chloride was given at a rate of 2 mL/min into the lumen (Figure 2). The pressure at the moment of the first leakage observed from the suture line was recorded as the blow-out pressure. All measurements were done *in situ*. After the measurements, tissue samples were taken from the liver and abdominal aorta for histopathological examination. The experiment was terminated by creating a pneumothorax.

In all groups, one centimeter segment of colon (including 0.5 cm proximal and 0.5 cm distal to the anastomotic line) was resected (Figure 3). The suture material was removed and the samples were kept in liquid nitrogen until the assay day.

Biochemical measurements were done as described previously^[14]. The volume of hydroxyproline was defined as micromoles of hydroxyproline per gram of wet weight.

Statistical analysis

Mann Whitney U test was used for statistical analysis compare the blow-out pressures and hydroxyproline contents between groups. $P < 0.05$ was considered statistically significant.

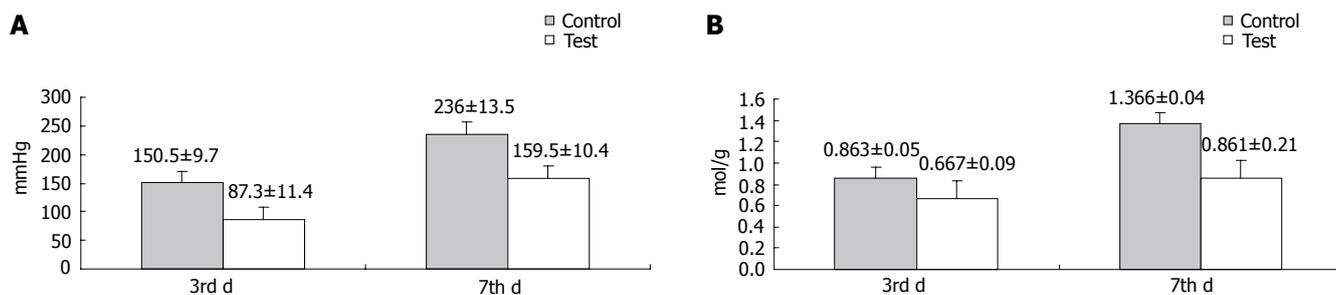


Figure 4 Mean blow-out pressure (A) and hydroxyproline level (B) in test and control groups.

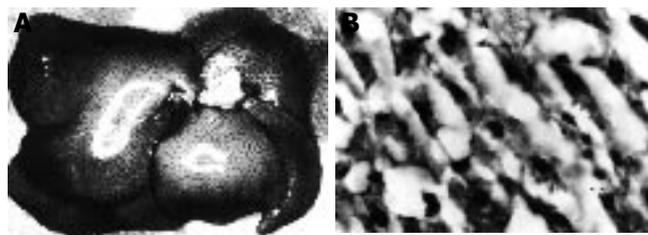


Figure 5 Macroscopic (A) and microscopic (B) steatosis in liver.

RESULTS

The rats in the test group gained significant weight (Table 1). The weight difference between the pre and post-feeding periods was statistically significant ($P < 0.05$).

After feeding with a high cholesterol containing diet for two months, a statistically significant difference serum cholesterol and triglyceride levels between the test and control groups was observed ($P < 0.05$, Figure 1). In the test group, serum total cholesterol level was 951 ± 72.4 mg/L and triglyceride level was 1425 ± 98.1 mg/L, while they were 423 ± 10.3 mg/L and 538 ± 23.6 mg/L in the control group, respectively.

On the third postoperative day, the mean blow-out pressure was 87.3 ± 11.4 mmHg in the test group and 150.5 ± 9.7 mmHg in the control group (Figure 4A). This difference was statistically significant ($P < 0.05$). The mean hydroxyproline level was 0.667 ± 0.09 in test group and 0.863 ± 0.05 in control group ($P < 0.05$) (Figure 4B).

On the seventh postoperative day, the blow-out pressure was 159.5 ± 10.4 mmHg in the test group and 236 ± 13.5 mmHg in the control group, while the hydroxyproline level was 0.861 ± 0.21 in the test group and 1.366 ± 0.06 in the control group, respectively. The difference in the parameters between the two groups was statistically significant ($P < 0.05$) (Figures 4A and 4B).

Liver tissue samples of the test group had a significant macroscopic steatosis compared to the control group (Figure 5A). In microscopic examination with hematoxylin-eosin staining, significant steatosis was also found in the test group (Figure 5B). No difference in microscopic results of the aortic specimens was found between the two groups.

DISCUSSION

In this study, left colon anastomosis was performed in hy-

percholesterolemic rats and the mechanical and biochemical parameters of anastomotic wound healing were investigated. Anastomotic wound healing in the test group was worse than that in the control group in terms of hydroxyproline levels and blow-out pressures. Significant steatosis was found in the liver specimens but no atherosclerotic changes in the aortic specimens. This finding is in accordance with several studies^[15-17].

Factors influencing colonic healing have been widely studied^[2,4]. Since blood flow is the major component of wound healing, hypercholesterolemia-induced atherosclerosis has a detrimental effect on intestinal anastomotic wound healing. However, the effects of hypercholesterolemia start long before the onset of atherosclerosis^[16-20]. There are also reports on how hypercholesterolemia worsens vascular functions using different pathways of microendocrine system inside the endothelium^[21-23]. Ross *et al.*^[24] have shown that monocytes accumulate on endothelium and damage it. Besides the macrovascular system, hypercholesterolemia also plays an important role in microvascular endothelial injury.

The cause of hypercholesterolemia-induced endothelial damage is predominantly determined by loss of endothelium-mediated relaxation. The mechanisms are as follows: decrease in the relaxing effect of NO/EDRF, ADP, thrombin, Ca^{++} -ionophore A 23187 on endothelium^[2,16,21,25,26]; increase in platelet aggregation and turnover as well as the sensitivity of platelets to aggregating substances due to the modification of effects of PGI₂ and TXA₂^[27-29]; increase in the release of endothelium-derived vasoconstrictor substances such as serotonin and TXA₂ through the cyclooxygenase pathway^[22,27].

The above effects have been shown *in vivo* and *in vitro* in experimental models of rabbit aorta, monkey iliac artery, pig coronary artery and human coronary arteries^[28]. Our study did not investigate these effects, but the effect of hypercholesterolemia on hydroxyproline level and blow-out pressure was emphasized.

Why hypercholesterolemia worsens anastomotic wound healing is still unknown. Luminal narrowing is the net result of atherosclerosis in both macro and microvascular systems. Consequently, ischemia and related injuries occur. Hypercholesterolemia is the major risk factor for atherosclerosis. In early phase of hypercholesterolemia, no morphological change takes place in arterial wall but vasospasm occurs due to neurohumoral mechanisms^[30]. Atherosclerosis is an irreversible process but hypercholesterolemia is reversible and these effects are preventable.

In our study, anastomotic wound healing in the test group was worse than that in the control group. Since hypercholesterolemia predominantly affects the endothelium, this can partly be explained as a result of endothelial damage-induced vasospasm. But we did not measure the anastomotic blood flow or assess the endothelial injury. Although a relationship between hypercholesterolemia and wound healing was demonstrated, our results could not fully explain the mechanisms. Further studies are needed to reveal the relationship between hypercholesterolemia and anastomotic wound healing.

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Cloning of α - β fusion gene from *Clostridium perfringens* and its expression

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Abstract

AIM: To study the cloning of α - β fusion gene from *Clostridium perfringens* and the immunogenicity of α - β fusion expression.

METHODS: Cloning was accomplished after PCR amplification from strains NCTC64609 and C58-1 of the protective antigen genes of α -toxin and β -toxin. The fragment of the gene was cloned using plasmid pZCPAB. This fragment coded for the gene with the stable expression of α - β fusion gene binding. In order to verify the exact location of the α - β fusion gene, domain plasmids were constructed. The two genes were fused into expression vector pBV221. The expressed α - β fusion protein was identified by ELISA, SDS-PAGE, Western blotting and neutralization assay.

RESULTS: The protective α -toxin gene (cpa906) and the β -toxin gene (cpb930) were obtained. The recombinant plasmid pZCPAB carrying α - β fusion gene was constructed and transformed into BL21(DE3). The recombinant strain BL21(DE3)(pZCPAB) was obtained. After the recombinant strain BL21(DE3)(pZCPAB) was induced by 42°C, its expressed product was about 22.14% of total cellular protein at SDS-PAGE and thin-layer gel scanning analysis. Neutralization assay indicated that the antibody induced by immunization with α - β fusion protein could neutralize the toxicity of α -toxin and β -toxin.

CONCLUSION: The obtained α -toxin and β -toxin genes are correct. The recombinant strain BL21(DE3)(pZCPAB) could produce α - β fusion protein. This protein can be used for immunization and is immunogenic. The antibody induced by immunization with α - β fusion protein could neutralize the toxicity of α -toxin and β -toxin.

INTRODUCTION

Clostridium perfringens is an anaerobe, forming a part of normal intestinal flora in humans and animals^[1]. This organism is the causative agent of diseases such as gas gangrene, hemorrhagic enteritis, enterotoxemia^[2]. It is also a secondary pathogen in various diseases, such as necrotic enteritis^[3]. *Clostridium perfringens* can produce extra cellular toxins and enzymes including α -toxin and β -toxin produced by *Clostridium perfringens* types A and C respectively. These toxins are significant virulence factors in case of gas gangrene or clostridial myonecrosis^[4,5].

In this study, α -toxin and β -toxin protective antigen genes were amplified by PCR from *Clostridium perfringens* type A strain NCTC64609 and type C strain C58-1 respectively. The recombinant plasmid pZCPAB carrying α - β fusion gene was constructed by transforming it into BL21(DE3). The fusion protein that could produce α - β was obtained. The expressed α - β fusion protein could resist the attack of α -toxin or β -toxin.

MATERIALS AND METHODS

Materials

Clostridium perfringens type A strain NCTC64609 and type C strain C58-1 were purchased from China Institute of Veterinary Drug Control. Plasmid pBV221 and *E coli* DH5 α , BL21(DE3) were kept in our Laboratory. Plasmid pMD18-T was purchased from TaKaRa Company. Taq DNA polymerase, T4 DNA ligase, restriction endonuclease and DNA markers DL2000 were purchased from TaKaRa Company. DNA fragment recover kit, SDS, IPTG, Rnase A and lysozyme were purchased from Promega Company. Other reagents if not described, were of analytic purity.

Extraction of genomic DNA of *clostridium perfringens*

Clostridium perfringens strains were incubated in TYG me-

dium at 37 °C overnight. Bacterium cells were harvested by centrifugation, dissolved in TE buffer containing lysozyme(2 g/L) and incubated at 37°C for 1 h. Then SDS(final concentration was 1%), EDTA(final concentration was 50 mmol/L) and proteinase K(final concentration was 150 mg/L) were added. The reaction mixture was incubated at 58 °C for 4 h and extracted three times with equal volume of saturated phenol. The aqueous phase was transferred to an Eppendorf tube. Then 0.2 volume of ammonium acetate(10 mol/L) and 2 volumes of ice-cold absolute alcohol were added, mixed well and deposited for 10 min at room temperature. Chromosome DNA deposition like clew was picked with a glass stick to another Eppendorf tube. Appropriate TE was added to dissolve DNA and stored at 4 °C.

PCR amplification of α -toxin and β -toxin protective antigen genes

Chromosome DNA of *Clostridium perfringens* was amplified by PCR from genomic DNA using forward primers designed from the available sequence.

One set of primers of α -toxin protective antigen gene (cpa906) was synthesized. The *EcoR* I and *BamH* I sites were inserted to the 5' and 3' ends of α -toxin genes via PCR. The sequence of α -toxin primers is as follows:

Up-stream primer:

P1: CCGGAATTCATGGGTCGGGATCCTGAT
EcoR I

Down-stream primer:

P2: GCGGATCCCTTAATTTATATTATA
BamH I

Another set of primers of β -toxin protective antigen gene was synthesized. The *BamH* I and *Pst* I sites were inserted to the 5' and 3' ends of β -toxin genes via PCR. The sequence of β -toxin primers is as follows:

Up-stream primer:

P1: 5'-CGCGGATCCAATGATATAGGTAAACT-3'
BamH I

Down-stream primer:

P2: 5'-CCGCTGCAGCTACTTAATAGCTGT-3'
Pst I

The following components were added into a 500 μ L microcentrifuge tube. Each PCR contained 0.1 μ mol/L primer1, 0.1 μ mol/L primer2, 0.25 mmol/L dNTP, 1.5 mmol/L MgCl₂, 10 μ L 10 \times PCR buffer and appropriate template DNA. Sterile distilled water was added into a 100 μ L reaction system. After the tubes were heated at 95 °C for 5 min, 5 u *Taq* DNA polymerase was added into each tube. Thirty cycles of PCR were performed (denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min). The amplified α - and β -toxin genes were purified separately by agarose gel electrophoresis to remove primers and extraneous amplification products.

Cloning and sequencing of α -toxin and β -toxin protective antigen genes

The α -toxin and β -toxin protective antigen genes were separated and purified by agarose gel electrophoresis alongside DNA marker DL2000 and sequentially ligated to the pMD18-T cloning vector. Sequencing was performed with ABI 377 sequencer from Takara Company.

Construction of expression vector carrying α - β fusion gene

The α -toxin protective antigen gene and expression plasmid pBV221 were digested by restrictive enzymes *EcoR* I and *BamH* I respectively to generate cohesive ends. The α -toxin gene was inserted into the vector pBV221 and the recombinant vector pZBVA1 containing α -toxin protective antigen genes of *Clostridium perfringens* was constructed. Then the β -toxin protective antigen gene and the recombinant vector pZBVA1 were digested by restrictive enzymes *BamH* I and *Pst* I respectively to generate cohesive ends. The β -toxin gene was inserted into the vector pZBVA1 and recombinant expression vector pZCPAB containing α - β fusion gene was obtained. The sequence of inserted fragment was confirmed by DNA sequencing.

Growth condition for α - β fusion gene expression

The recombinant was transformed into *E. coli* BL21(DE3) and selected by agar plate containing ampicillin and confirmed by restriction enzyme mapping. *E. coli* BL21(DE3) cells transformed with the plasmids described above were grown in LB medium with 50 mg/mL ampicillin (1/50) at 30 °C to OD₆₀₀=0.4~0.6. The growth was conditioned at 42 °C for 5 h in order to express α - β fusion protein.

Analysis of bacterial samples

The cultured cells were collected by centrifugation at 5000 r/min for 10 min at 4 °C. The optimum time of maximum expression of proteins was analyzed through SDS-PAGE. The expressed protein was tested through Western blot with specific antiserum. SDS-PAGE and Western blotting were performed as previously described^[6,7]. The antigen-binding activity of the α - β fusion protein was determined by ELISA as previously described^[8]. For determination of protein expression, the absorption value at 560 nm was measured by thin-layer chromatogram scanner type CS-9000.

Protective effect of α - β fusion protein on mice

The 8-wk old Kunming mice were divided into 8 groups randomly, 10 mice in each group. In treatment groups, each mouse was inoculated with inactive recombinant strains that could express α - β fusion protein. In control groups, each mouse was inoculated with strains that could not express α - β fusion protein. After 4 wk, each mouse of 4 groups was treated with one minimum lethal dose (MLD) of α -toxin or β -toxin. Another 4 groups were treated with 2 MLD of α -toxin or β -toxin. The survived mice/total mice were counted in a week. The segments of liver, lung, heart, spleen and intestines of mice in treatment group and control group were tested by LM assay.

RESULTS

Gene cloning and identification of α -toxin gene from *clostridium perfringens* type A

The α -toxin gene from *Clostridium perfringens* type A strain NCTC64609 was isolated from genomic DNA-extracted template by PCR amplification. The reaction yielded a single product. Electrophoresis of PCR products confirmed

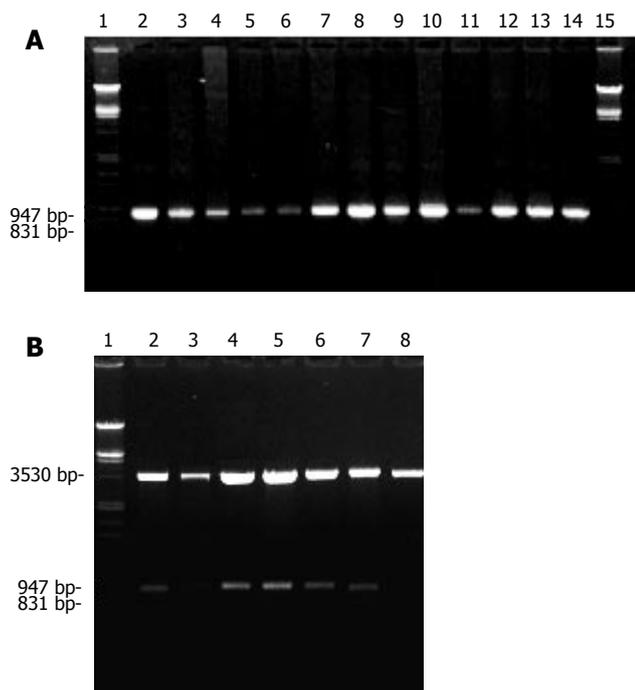


Figure 1 Identification of α -toxin gene product amplified by PCR (A) and recombinant plasmid by enzyme digestion (B). In A, lanes 1, 15: DNA markers; lanes 2-14: product of α -toxin gene amplified by PCR; In B, lane 1: DNA markers; lanes 2-7: pMD 906/ EcoR I + BamH I; lane 8: pMD 906/ EcoR I.

ATGGGTCGGGATCCTGATACAGATAATTTCTCAAAGGATAATAGTTGGTATTTAGCT
TATTCTATACCTGACACAGGGGAATCACAATAAGAAAATTTTCAGCATTAGCTAGA
TATGAATGGCAAAGAGGAACTATAACAAGCTACATTCTATCTTGGAGAGGCTATG
CACTATTTTGGAGATATAGATACTCCATATCATCTGCTAATGTTACTGCCGTTGATA
GCGCAGGACATGTTAAGTTTGAGACTTTTGCAAGAGGAAAGAAAAGAACAGTATAA
AATAAACACAGCAGGTTGCAAACTATGAGGCTTTTTATACTGATATCTTAAAAACA
AAGATTTAATGCATGGTCAAAGAATATGCAAGAGGTTTTGCTAAAACAGGAAAAT
CAATATACTATAGTCATGCTAGCATGCATAGTTGGGATGATTGGGATTATGCAGCAA
AGGTAACCTTTAGCTAACTCTCAAAAAGGAACAGCGGGATATATTTATAGATCTTACA
CGATGTATCAGAGGGTAATGATCCATCAGTTGGAAAGAAATGTAAAAGAACTAGTAG
CTTACATATCAACTAGTGGTGAGAAAGATGCTGGAACAGATGACTACATGATTTTTG
GAATCAAAAACAAAGGATGAAAAACTCAAGAATGGGAAATGGACAACCCAGGAAAT
GATTTTATGACTGGAAGTAAAGACACTTATACTTTCAAATTAAGATGAAAATCTAA
AAATTGATGATATACAAAATATGTGGATTAGAAAAGAAAATATACAGCATTCTCAGAT
GCTTATAAGCCAGAAAACATAAAGATAATAGCAAATGGAAAAGTTGTAGTGACAAA
GATATAACGAGTGGATTTCAGGAAATCAACTTATAATATAAAATAA

Figure 2 Nucleotide sequence of α -toxin gene from *Clostridium perfringens* type A strain NCTC64609.

the length of PCR fragment, which was approximately 900 bp (Figure 1A). The α -toxin gene was purified with DNA fragment recover kit and evaluated by agarose gel electrophoresis. The α -toxin gene fragment was ligated to the cloning vector pMD18-T with T4 DNA ligase. The cloning vector containing the α -toxin gene was introduced into competent *E. coli* DH5 α cells by CaCl_2 transformation. Transformed *E. coli* were grown at 37°C in medium containing X-gal/IPTG and ampicillin. The positive plasmids were identified via sequential digestion with *EcoR* I and *BamH* I and α -toxin protective antigen gene about 900 bp was obtained (Figure 1B). Sequence of inserted DNA was analyzed with automatic sequence analyzer and about 906 bp in size and encoded 302 amino acid residues (Figure 2).

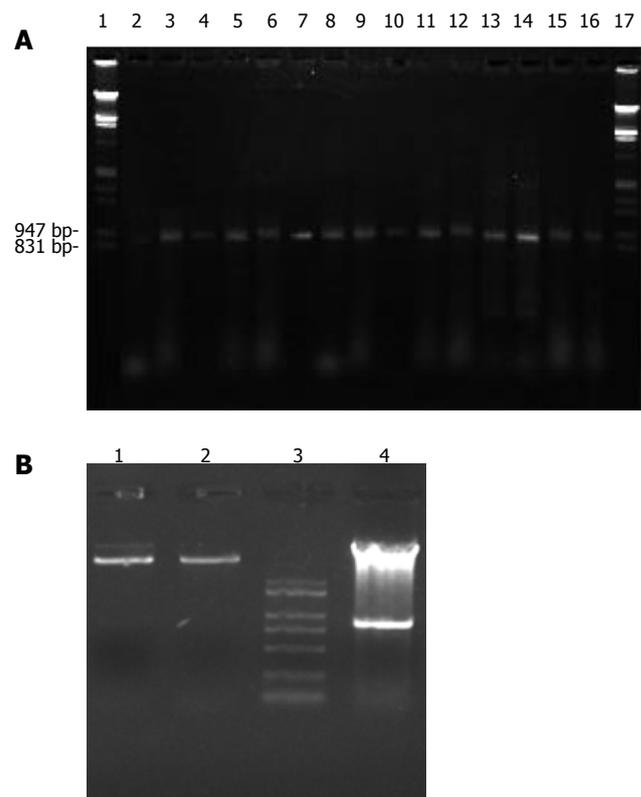


Figure 3 Identification of β -toxin gene product amplified by PCR (A) and recombinant plasmid by enzyme digestion (B). In A, lanes 1,17: DNA markers; lanes 2-16: product of β -toxin gene amplified by PCR; In B, lane 1: pMD930/EcoR I; lane 2: pMD930/ Pst I; lane 3: DNA Markers; lane 4: pMD930/BamH I+Pst I

The recombinant plasmid was named pMD 906.

Cloning and identification of β -toxin gene from *clostridium perfringens* type A

The β -toxin protective antigen genes were amplified from *Clostridium perfringens* type C and strain C58-1 by PCR. The PCR products were approximately 930 bp in length (Figure 3A). The β -toxin gene was purified and ligated to the cloning vector pMD18-T as α -toxin gene. The positive plasmids were identified via sequential digestion with *Pst* I and *BamH* I and about 930 bp β -toxin genes were obtained (Figure 3B). Sequence of inserted DNA was analyzed with automatic sequence analyzer and about 930 bp in length and encoded 310 amino acid residues (Figure 4). The recombinant plasmid was named pMD 930.

Construction of recombinant expression vector pZCPAB

The recombinant expression plasmids containing α - β fusion gene were constructed. The positive plasmids were identified via sequential digestion with *EcoR* I and *Pst* I and about 1.8 kb α - β fusion genes were obtained (Figure 5). Sequence of α - β fusion genes was analyzed with automatic sequence analyzer and about 1842 bp in length and encoded 614 amino acid residues. The recombinant plasmid was named pZCPAB.

Expression of α - β fusion gene in *E coli* BL21 (DE3) (PZCPAB)

The recombinant plasmid pZCPAB carrying α - β fusion gene was transformed into *E coli* BL21(DE3) and the

AATGATATAGGTAAACTACTACTATACTAGAAATAAGACATCAGATGGCTACTACT
 ATAATTACACAAAATGATAAACAGATAATATCATATCAATCTGTTGACTCTTCAAGT
 AAAAAATGAAGATGGTTTTACTGCATCTATAGATGCTAGATTTATCGATGATAAATAT
 TCATCTGAAATGACAACCTTTAATAAACTTAACCTGATTTATGTCTTCAAAAAAGAA
 GATGTTATAAAAAATACAATTTGCATGATGTTACTAATTCTACTGCAATTAATTTTC
 CGGTTAGATACTCGATTTCTATTTTAAATGAAAGTATTAATGAAAATGTAAAAATAG
 TTGATAGTATTCTAAAAATACAATTTCTCAAAAACTGTATCCAATACAATGGGAT
 ACAAAAATAGGAGGTTCAATTGAAATAGAAAAATAAACCTAAAGCTTCAATTGAAA
 GCGAATATGCTGAATCATCTACAATAGAATATGTCCAACCTGATTTTCTACTATACA
 GACAGATCATTCAACCTCTAAAGCTTCATGGGATACAAAATTTACAGAACTACTCG
 TGGTAATTATAATTTAAATCAAAACAACCTGTATATGAAATGAAATGTTTATGTAC
 GGAAGATATACTAATGTTCTGCAACTGAAAATATAATCCAGATTATCAAATGTCA
 AAATAATAACAGGTGGTTTAAACCCTAATATGTCTGTAGTTCTAACTGCTCCTAAT
 GGTACTGAAGAATCTATAATAAAAGTTAAATGGAGCGTGAAGAAACTGTTATTA
 TCTTAATTGGAATGTTGCTAACTGGGTAGGACAAGTCTATTCCAGGCTAGCTTTTGA
 TACCCCAAATGTAGATAGTCATATTTACATTCAAAATAAATTGGCTTACTCACAAA
 GTAACAGCTATTAAGTAG

Figure 4 Nucleotide sequence of β -toxin gene from *Clostridium perfringens* type C strain C58-1

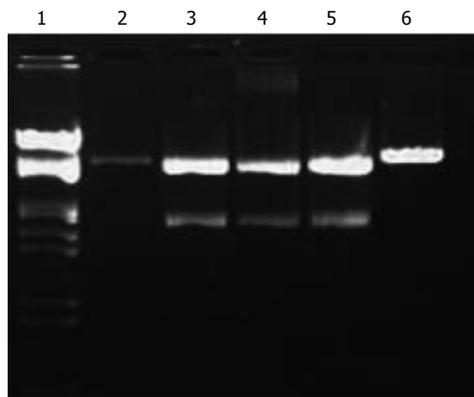


Figure 5 Agarose gel electrophoresis of restriction endonucleases-digested plasmid pZCPAB. Lane 1: DNA markers; lane 2: pZCPAB / EcoR I; lanes 3-5: pZCPAB / EcoR I+Pst I; lane 6: pZCPAB / BamH I.

recombinant strain BL21(DE3)(pZCPAB) was obtained. SDS-PAGE and Western blotting of the bacterial protein showed that a specific protein band with a molecular weight of 66 kD emerged in the bacterial protein of *E. coli* BL21(DE3)(pZCPAB). SDS-PAGE gel-scanning showed that the expressed α - β fusion protein accounted for 22.14% of the total bacterial protein (Figure 6). ELISA results indicated that the expressed fusion protein could be recognized by anti- α toxin antibody and anti- β toxin antibody respectively (Table 1).

Bioactivity assay of expressed α - β fusion protein

The expressed α - β fusion protein yielded in bacteria could provide protection of mice against the challenge of lethal doses of α -toxin or β -toxin of *Clostridium perfringens* (Table 2).

Pathological changes in different organs

The segments of liver, lung, heart, spleen and intestine were sampled from the control group in which mice died of toxicosis and the treatment group in which survived mice were immunized with α - β fusion protein. Then they were observed by LM assay.

In treatment group, the structure of liver was seen

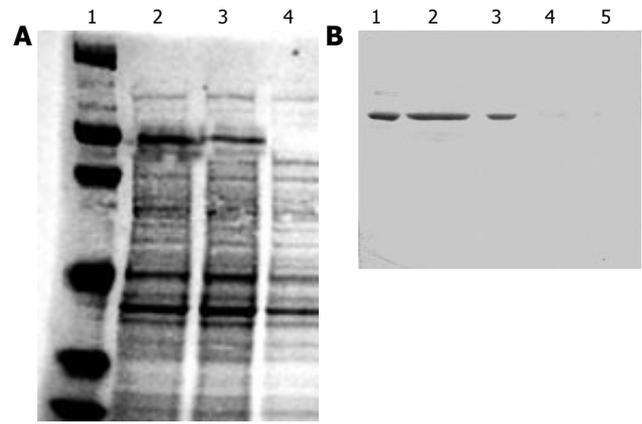


Figure 6 Analysis of expressed products of α - β fusion gene in *E. coli* by SDS-PAGE (A) and Western blot (B) In A, lane 1: protein markers (97400, 66200, 43000, 31000, 20100, 14400); lanes 2, 3: BL21(DE3)(pZCPAB); lane 4: BL21(DE3)(pBV221); In B, lane 1: BL21(DE3)(pZCPAB)/ α ; lane 2: BL21(DE3)(pZCPAB)/ α + β ; lane 3: BL21(DE3)(pZCPAB)/ β ; lanes 4, 5: BL21(DE3)(pBV221).

Table 1 Detection of the fusion protein by ELISA assay

| Antibody | pZCPAB | pBV221 | pXETA | pXETB |
|-----------------------|-----------|--------|-----------|-----------|
| Anti- α -toxin | 0.66±0.08 | 0.001 | 0.92±0.13 | |
| Anti- β -toxin | 0.72±0.12 | 0.002 | | 0.97±0.19 |

Table 2 Expressed α - β fusion protein in mice

| Challenge dose | α -toxin(survived mice/total mice) | | β - toxin(survived mice/total mice) | |
|----------------|---|-----------------|---|-----------------|
| | Control group | Treatment group | Control group | Treatment group |
| 1 ML | 0/10 | 10/10 | 0/10 | 10/10 |
| 2 ML | 0/10 | 8/10 | 0/10 | 9/10 |

clearly except that a few central veins of hepatic lobules were full of blood. The liver cytoarchitectures were intact (Figure 7A). In control group, the structure of hepatocytes was grain-like metamorphic. The nuclei of hepatocytes were concentrated and collapsed. Swollen hepatic sinusoids, veins of hepatic lobules and central veins were full of red cells or red thrombi, swollen Kupffer cells in the margin of hepatic sinusoids (Figure 7B).

The capillaries in alveolar septum of the lung in treatment group were slightly bloodshot with no other abnormalities (Figure 7C). The swollen capillaries in alveolar septum were full of red cells. Some of capillaries were full of red thrombi (Figure 7D).

The structure of cardiac muscle fibers in treatment group was intact (Figure 7E). The capillary vessels and cardiac muscle fibres were swollen and bloodshot (Figure 7F).

There was no abnormal structure of spleen in treatment group (Figure 7G). The splenic cord was extruded and could not be seen clearly (Figure 7H).

The structure of duodenum and jejunum in treatment group was intact (Figure 7I). The capillary vessels of lamina propria of tunica mucosa had little bleeding. The epithelia of tunica mucosa were metamorphic and

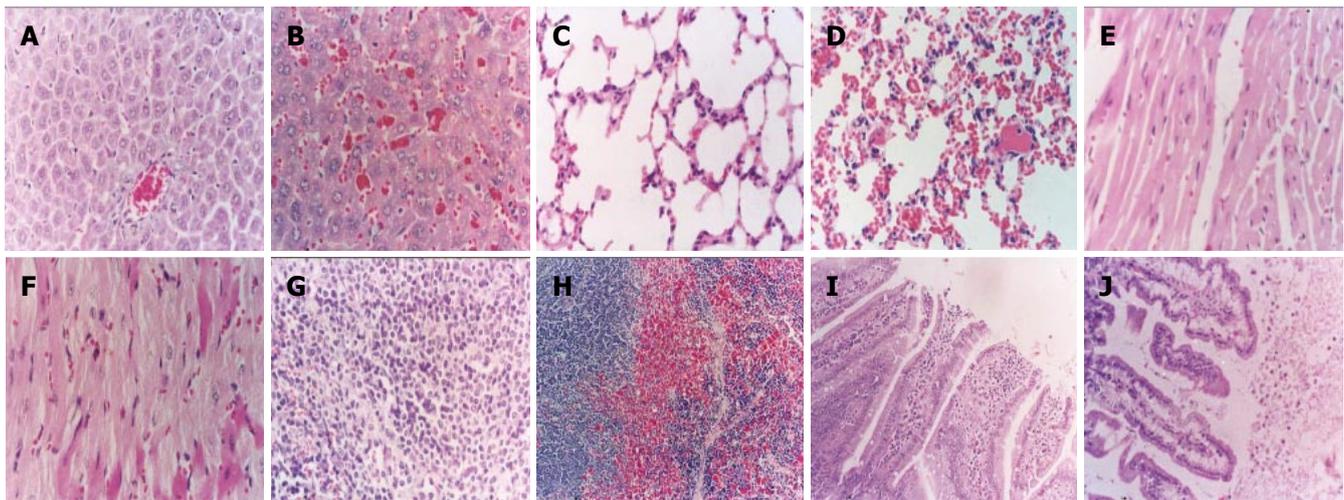


Figure 7 Pathological changes in livers of survived mice (A) and dead mice (B), in lung of survived mice (C) and dead mice (D), in heart of survived mice (E) and dead mice (F), in spleen of survived mice (G) and dead mice (H), in intestine of survived mice (I) and dead mice (J).

necrotic, one or many of intestinal villi fell into the enterocoele (Figure 7J).

DISCUSSION

Clostridium perfringens is ubiquitous in the environment and has been found in soil, decaying organic matter and a member of the gut flora in humans and animals. Different strains of *C. perfringens* can be classed into one of the five biotypes (A-E) according to the spectrum of toxins produced^[9]. *Clostridium perfringens* is a Gram-positive anaerobic spore-forming bacterium which is widely found in the environment and also in the intestinal tract of humans and animals. Due to the production of several exotoxins, *C. perfringens* is of great importance as a pathogen in humans, domestic animals and wildlife^[10]. The production of four major toxins alpha (α), beta (β), epsilon (ϵ) and iota (ι) allows the discrimination into five types from A to E. In veterinary medicine, each of these types has been linked to specific diseases^[11]. *Clostridium perfringens* enterotoxin (CPE) serves as an additional virulence factor which is believed to cause enteritis in pigs, cats and dogs^[12]. The Gram-positive pathogen *Clostridium perfringens* is a major cause of human and veterinary enteric disease largely because this bacterium can produce several toxins when present inside the gastrointestinal tract^[13].

Biotype A strains of *Clostridium perfringens* are of particular importance as the aetiological agents of gas gangrene in humans. Gangrenous disease is of increasing significance in elderly and diabetic population, especially in those who have undergone lower limb surgery, where impaired blood supply to tissues can lead to anoxic conditions suitable for proliferation of bacteria^[14]. The disease can also arise in patients who have undergone surgery of the gastrointestinal tract when contamination of damaged tissues with gut contents can result in the establishment of disease^[15]. *Clostridium perfringens* β -toxin is known to be the primary pathogenic factor of necrotic enteritis in type C strains that produce β -toxin. Necrotizing enteritis is believed to be due to the production of β -toxin by *Clostridium perfringens* type C. *Clostridium perfringens* β -toxin can

also cause dermonecrosis and oedema in the dorsal skin of animals^[16-18].

This paper describes the successful isolation and cloning of α -toxin and β -toxin genes from the strains NCTC64609 and C58-1 of *Clostridium perfringens* respectively. The construction and expression of α - β fusion gene, and the biological activities of α - β fusion protein have a significant impact on mice. To our knowledge, this is the first successful experiment of the development of a recombinant α - β fusion protein. We believe that it could serve as a model for development and construction of novel fusion protein for α -toxin, β -toxin and other potential toxins. In this study, the expressed α - β fusion protein could provide protection of mice against the challenge of lethal dose of α -toxin and β -toxin of *Clostridium perfringens*. These findings indicate that the expressed α - β fusion protein can be applied in preventing toxicosis of α -toxin and β -toxin of *Clostridium perfringens*.

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Prior appendectomy and the phenotype and course of Crohn's disease

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<http://www.wjgnet.com/1007-9327/12/1235.asp>

Abstract

AIM: To determine whether prior appendectomy modifies the phenotype and severity of Crohn's disease.

METHODS: Appendectomy status and smoking habits were specified by direct interview in 2838 patients consecutively seen between 1995 and 2004. Occurrence of complications and therapeutic needs were reviewed retrospectively. Additionally, annual disease activity was assessed prospectively between 1995 and 2004 in patients who had not had ileocecal resection and of a matched control group.

RESULTS: Compared to 1770 non-appendectomized patients, appendectomized patients more than 5 years before Crohn's disease diagnosis ($n=716$) were more often females, smokers, with ileal disease. Cox regression showed that prior appendectomy was positively related to the risk of intestinal stricture (adjusted hazard ratio, 1.24; 95% confidence interval, 1.13 to 1.36; $P=0.02$) and inversely related to the risk of perianal fistulization (adjusted hazard ratio, 0.75; 95% confidence interval, 0.68 to 0.83; $P=0.002$). No difference was observed between the two groups regarding the therapeutic needs, except for an increased risk of surgery in appendectomized patients, attributable to the increased prevalence of ileal disease. Between 1995 and 2004, Crohn's disease was active during 50% of years in appendectomized patients (1318 out of 2637 patient-years) and 51% in non-appendectomized patients (1454 out of 2841 patient-years; NS).

CONCLUSION: Prior appendectomy is associated with a more proximal disease and has an increased risk of stricture and a lesser risk of anal fistulization. However, the severity of the disease is unaffected.

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Key words: Crohn's disease; Appendectomy; Surgery; Smoking

INTRODUCTION

Two common environmental factors, cigarette smoking and appendectomy, have been found to play a role in inflammatory bowel diseases (IBD). Smoking has a role both in disease onset and disease course, and it is well established that it has opposite effects on the two diseases, beneficial in ulcerative colitis (UC) and deleterious in Crohn's disease^[1,2]. Previous appendectomy has a favourable effect on UC. Patients who have been appendectomized have a lesser risk of developing UC. Moreover, in the few appendectomized patients who develop UC, disease course is less severe, with a decreased need of colectomy compared to non-appendectomized patients^[3,4]. Of note, the effects of smoking and appendectomy are additive^[4]. In Crohn's disease, the effect of previous appendectomy remains debated. Some series reported an increased risk of Crohn's disease after appendectomy^[5-7], and others did not^[8-11]. These discrepancies may be partly linked to the inclusion or not of appendectomies performed close to the time of diagnosis. However, the largest study to date showed that the risk of Crohn's disease is increased up to 20 years after appendectomy^[7]. Data concerning the effect of appendectomy on the clinical course of Crohn's disease are scarce and contradictory, one study reporting no effect^[3] and another suggesting that previous appendectomy was associated with an increased risk of surgery^[12]. In the study by Andersson *et al*^[7], an increased risk of surgery for Crohn's disease was observed only in patients with perforated appendicitis.

The aim of the present study was to analyse the phenotype and clinical course of Crohn's disease in a large cohort of patients subjected to appendectomy compared with non-appendectomized patients.

MATERIALS AND METHODS

Patient population

From January 1995 to December 2004, all consecutive patients with Crohn's disease who came to our unit were

included in the study. The diagnostic criteria for Crohn's disease were those of Lennard-Jones^[13]

Appendectomy status and smoking habits

Appendectomy and smoking status were specified during a direct interview of the patients. The date of appendectomy was noted and patients were classified according to the time span between appendectomy and diagnosis. Patients were classified as smokers if they had smoked more than 7 cigarettes per week for at least six months during the six months preceding diagnosis of Crohn's disease and/or thereafter^[14].

Characteristics of Crohn's disease

The characteristics of Crohn's disease were completed according to the retrospective analysis of medical charts. The time of diagnosis was defined as the date of first detection of unequivocal inflammatory abnormalities of the intestine, as assessed from radiological, or endoscopic, or peroperative observations. The initial location of Crohn's disease lesions was determined by colonoscopy and small bowel X-ray. After diagnosis, patients were followed clinically with 3 to 4 visits per year, and only investigated again in case of flare-up or development of new symptoms. Morphological investigations included proctosigmoidoscopy, colonoscopy, and small bowel X-ray. Upper gastrointestinal endoscopy was only performed in case of gastroesophageal symptoms. Subjects were explored for ano-perineal disease at each visit.

Location and behavior of Crohn's disease were classified according to the Vienna classification^[15]. First morphological demonstration of narrowing or penetrating complication was used to date the occurrence of the complication defining behavior^[16].

Treatment of Crohn's disease

Our treatment policy has been exposed elsewhere^[16]. Flare-up episodes were treated with mesalamine (3-4 g daily) or prednisolone (1 mg/kg per day, progressively tapered after four weeks), according to the clinical severity. When steroid therapy failed, patients seen before 1999 were given a 3-wk course of enteral or parenteral nutrition; those seen after June 1999, when Infliximab became available in France, received Infliximab 5 mg/kg.

As maintenance treatment, we used aminosalicylates (sulphasalazine, olsalazine or mesalamine, 2-3 g daily) for asymptomatic or moderately active forms of the disease, and immunosuppressive drugs for severe forms (steroid-dependent or poorly responsive to steroids). Azathioprine 2 mg/kg per day was used as first line immunosuppressive drug. In case of repeated flare-ups or chronic active disease in a patient receiving azathioprine, its dosage was increased to 2.5-3 mg/kg per day. Intramuscular methotrexate (20-25 mg weekly) was used in patients unresponsive or intolerant to azathioprine. Its dosage was tapered progressively to 10-15 mg, and re-augmented in case of clinical relapse.

Although the overall strategy remained mostly unchanged, there was a clear tendency over time to initiate immunosuppressants earlier in the disease course. Surgery

was reserved for stenotic and extra-parietal complications, or intractable forms after a well-conducted medical management.

Phenotype and severity of Crohn's disease

Phenotyping Crohn's disease took into account disease location and the occurrence of a stricturing or penetrating complication. Overall severity of the disease was assessed in two ways: first retrospectively, taking into account the importance of the medical therapy, i.e. need for glucocorticoid, nutritional support, immunosuppressive drugs, and Infliximab, and finally incidence of excisional surgery. Second, patients who had not had ileo-cecal resection prior to inclusion were followed-up prospectively from the date of inclusion to December 2004, and activity of the disease was assessed prospectively by analyzing the occurrence of a flare-up each year. A patient-year was considered as active if a flare-up or a complication occurred during the year, and inactive otherwise.

Statistics

Continuous data are expressed as mean (standard deviation), and differences between the groups were tested for significance by Student's *t* test. Discrete data are given as percentages, and comparisons were made with Pearson's Chi-square test.

The retrospective study analyzed the effect of prior appendectomy on the long-term course of Crohn's disease. For this purpose, non-appendectomized patients were included in the control group until the time an ileo-cecal resection was eventually performed. To avoid biases related to the effect of ileo-cecal resection on the subsequent evolution of Crohn's disease, appendectomized patients were also censored at the time of ileo-cecal resection. For actuarial analysis, the Kaplan-Meier model was used, with the date of diagnosis as starting point. The curves were compared by the Log-rank test. Multivariate analyses were performed with Cox proportional hazards regression to adjust for confounding. All baseline variables suspected to be possible predictors of complication or intestinal surgery [young age (< 20 years), old age (equal to or above 40 years), gender, ethnicity (Caucasian or not), socioeconomic status (high or low-moderate), diagnosis after 1987, familial history, extra-intestinal manifestations, smoking status, initial disease location (esophago-gastro-duodenal, jejunal, ileal, colonic, and anoperineal lesions)], were entered into the model. Diagnosis after 1987 was retained as a variable because the use of immunosuppressive therapy became frequent since that year. Results of analysis are presented as hazard ratios (HRs) with 95% confidence intervals.

The prospective analysis included consecutive Crohn's disease patients seen between 1995 and 2004 who had had appendectomy but no ileo-cecal resection before 1995. Those patients were matched for sex, birth date (boxes of five years), date of diagnosis (boxes of five years), and Vienna classification disease location, with non-appendectomized patients without previous ileo-cecal resection. Patients of both groups were censored at the time of ileo-cecal resection. Calculations were performed using GB-stat statistical software.

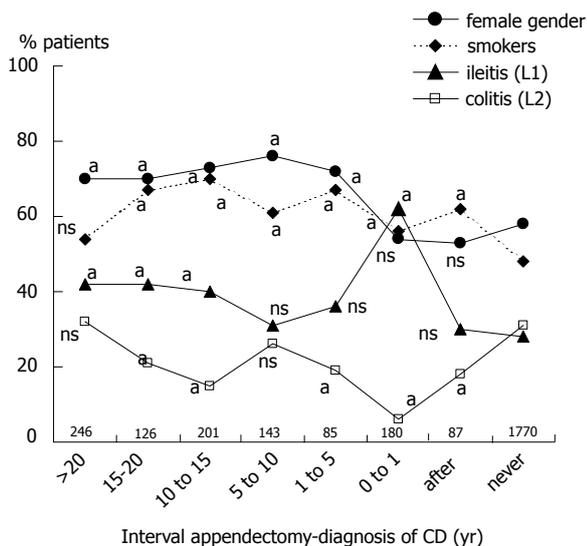


Figure 1 Characteristics of patients and Crohn's disease (given as percentages) according to appendectomy status and the time span between appendectomy and diagnosis of Crohn's disease. Numbers indicate the number of patients in each group. ^a and ns represent the *P* value of the comparison between the subgroup and the group of never appendectomized patients (^a*P* < 0.05; ns: not significant).

RESULTS

Appendectomy in relation to disease onset

Among 2926 patients with Crohn's disease, 1068 patients (38% of those with appendectomy status known) had been appendectomized, including 87 after Crohn's disease diagnosis. Compared to non-appendectomized patients, the 981 appendectomized patients before or at diagnosis were more often females (69 *vs* 58%, *P* < 0.001), Caucasians (90 *vs* 84%, *P* < 0.001), and active smokers (61 *vs* 48%, *P* < 0.001). They were also older at diagnosis (29.5 ± 12.1 *vs* 27.4 ± 12.7 years, *P* < 0.001), and the time span between first symptom attributable retrospectively to Crohn's disease and Crohn's disease diagnosis was longer (21.9 ± 48.9 *vs* 16.5 ± 38.3 months in non-appendectomized patients, *P* = 0.003). Disease location differed significantly between the two groups, with a predominance of ileitis (L1 location) counterbalanced by a decrease of colitis (L2 location) in appendectomized patients (43 and 20%, *vs* 28 and 31%, respectively, in non-appendectomized patients, *P* < 0.001), without significant differences in the proportion of ileocolitis (L3) and upper digestive tract location (L4). Important differences were seen regarding gender distribution and disease location according to the time span between appendectomy and Crohn's disease diagnosis (Figure 1). A significant female predominance was observed only in patients appendectomized more than one year prior to Crohn's disease diagnosis. L1 was the main disease location whatever the interval between appendectomy and Crohn's disease diagnosis, however this increase was particularly marked in patients appendectomized at or within one year preceding the diagnosis. Compared to this latter subgroup, subgroups of appendectomized patients who had had appendectomy at various intervals prior to Crohn's disease differed significantly by a higher proportion of females, a lower prevalence of L1 location and a higher prevalence

Table 1 Main characteristics of Crohn's disease in non-appendectomized and appendectomized patients

| | Non-appendectomized | Appendectomized | <i>P</i> |
|---------------------------------------|---------------------|-----------------|----------|
| Number of patients | 1770 | 716 | |
| Female gender | 1019 (58) | 515 (72) | 0.001 |
| Age at diagnosis (yr) | 27.5 ± 12.8 | 32.1 ± 13 | 0.001 |
| Duration of disease (yr) ¹ | 9.6 ± 8.6 | 9.1 ± 8.1 | NS |
| Caucasian ethnicity | 1484 (84) | 663 (93) | 0.001 |
| High socio-economic status | 535 (30) | 171 (24) | 0.001 |
| Disease onset after 1987 | 1374 (78) | 554 (77) | NS |
| Familial history | 272 (15) | 79 (11) | 0.004 |
| Current smokers | 856 (48) | 444 (62) | 0.001 |
| Disease location | | | |
| Terminal ileum (L1) | 489 (28) | 277 (39) | |
| Colon (L2) | 548 (31) | 173 (24) | 0.001 |
| Ileocolon (L3) | 514 (29) | 187 (26) | |
| Upper gastrointestinal (L4) | 219 (12) | 79 (11) | |
| Extra-intestinal manifestations | 1136 (64) | 422 (59) | 0.01 |

¹ at last visit or when censored

Number in parentheses is percentages.

of L2 location. Finally, there was a higher proportion of smokers in appendectomized patients whatever the date of appendectomy. To avoid inclusion of patients for whom appendectomy could have been performed for a missed diagnosis of Crohn's disease, we excluded from further analysis, in addition to the 180 patients appendectomized within the year of diagnosis, the 85 patients in whom appendectomy had been performed within the five years before disease onset. Thus 716 patients were included in the retrospective analysis (Figure 2). Among those patients, appendectomy had been performed 5 to 76 years (median, 16 years) before diagnosis of Crohn's disease, at a median age of 11 years (range 0-67).

Retrospective analysis

The comparison of these 716 appendectomized patients with non-appendectomized patients is given in Table 1. The results were similar to the whole group of appendectomized patients, with a Crohn's disease diagnosis later in life and a predominance of both women and active smokers. In addition, appendectomized patients were more often Caucasian, of low-to-moderate socioeconomic status, without family history, and had less extra-intestinal manifestations. The most frequent disease location according to Vienna classification was ileum only in appendectomized patients, whereas it was colon only in non-appendectomized patients. More detailed comparison of initial and cumulative disease location revealed that prior appendectomy was associated with a higher prevalence of ileal involvement, while distal colon, rectum, and anus were more frequently spared (Figure 3). Cumulative disease behavior was inflammatory (B1) in 46%, stricturing (B2) in 15%, and penetrating (B3) in 39% of appendectomized patients, *vs* 43% (B1), 11% (B2), and 46% (B3), respectively, in non-appendectomized patients. Because disease behavior is highly dependent on disease location

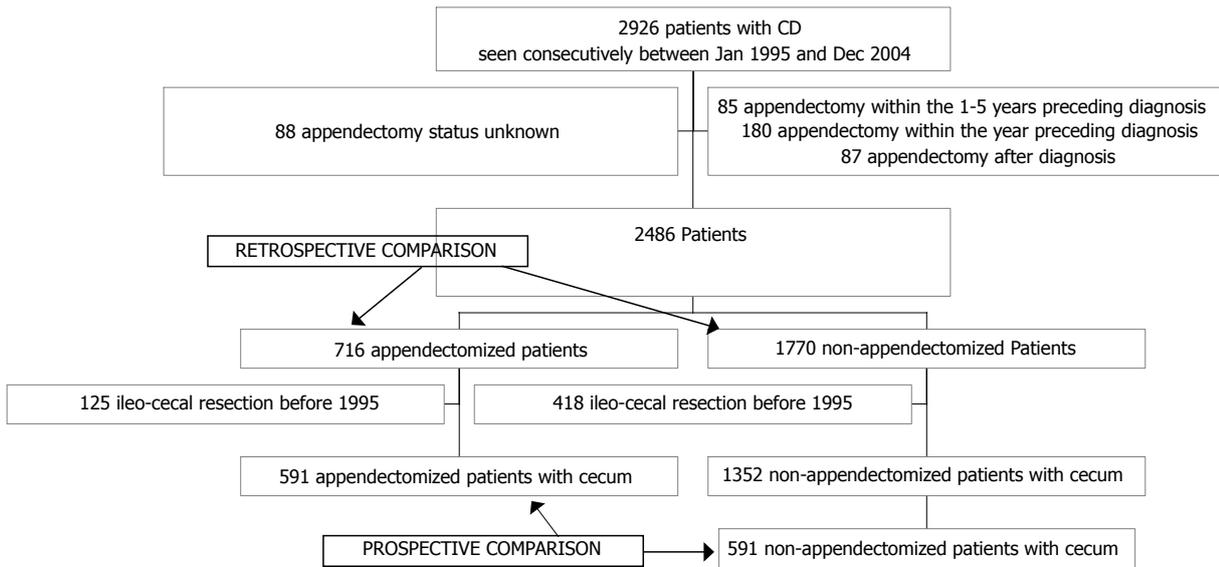


Figure 2 Flow chart of patients studied.

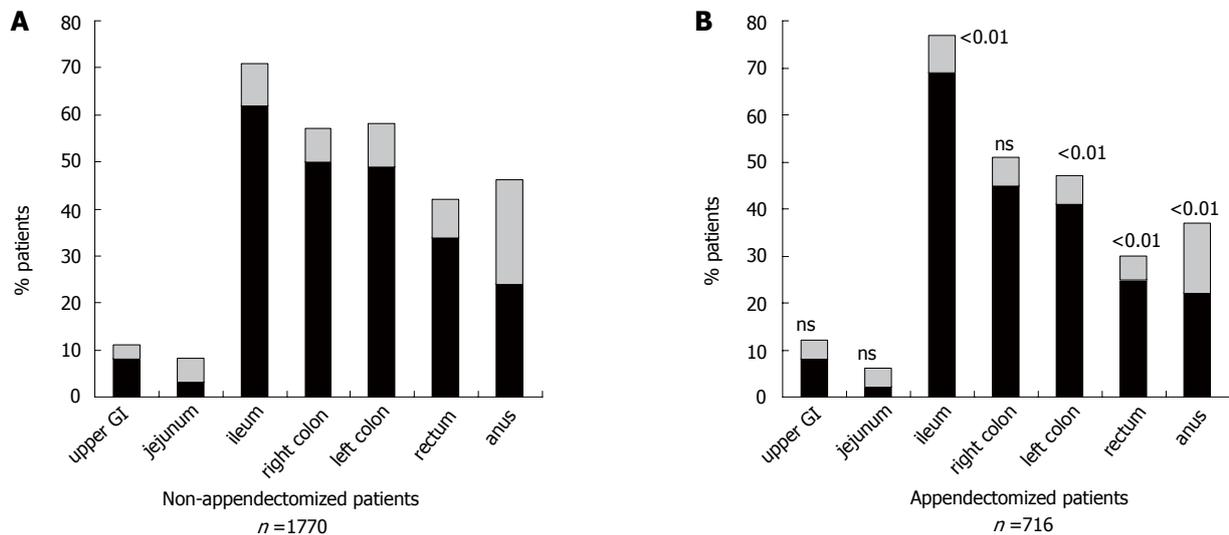


Figure 3 Initial and cumulative Crohn's disease involvement of various segments of the digestive tract in non-appendectomized and appendectomized patients. The numbers above the columns indicate the P values comparing the frequencies of cumulative involvement of each segment between the two groups.

and duration^[16], the respective risks of stricture, intestinal perforation, and perianal perforation were calculated in each location group cumulatively (Table 2). These calculations demonstrated in appendectomized compared to non-appendectomized patients an increased risk of stricture in the L1 group, and a decreased risk of perianal perforating disease in the L2 group. Cox analysis in the whole population pooling the four location groups (2486 patients) confirmed that prior appendectomy was positively related to the risk of intestinal stricture (adjusted hazard ratio, 1.24; 95% confidence interval, 1.13 to 1.36; $P=0.02$) and inversely related to the risk of perianal perforation (adjusted hazard ratio, 0.75; 95% confidence interval, 0.68 to 0.83; $P=0.002$). The respective proportions of appendectomized and non-appendectomized patients who required oral steroids, enteral or parenteral nutrition, immunosuppressive therapy, infliximab, and

surgery were not significantly different in the whole population nor in each location group (Table 3). In the whole population, the 10-yr cumulative need for first excisional surgery was significantly higher in appendectomized vs non-appendectomized patients ($54 \pm 2\%$ vs $48 \pm 2\%$, respectively; $P=0.02$). However, multivariate analysis selected as predictive factors of surgery young age (adjusted hazard ratio, 0.80; 95% confidence interval, 0.74 to 0.86; $P=0.002$), ileal involvement (adjusted hazard ratio, 1.73; 95% confidence interval, 1.60 to 1.87; $P<0.001$) and colonic involvement (adjusted hazard ratio, 0.66; 95% confidence interval, 0.62 to 0.71; $P<0.001$) and did not confirm the specific role of appendectomy ($P=0.33$). Within each location group, the 10-yr cumulative need for first excisional surgery did not differ significantly (L1 $74 \pm 4\%$ vs $67 \pm 3\%$; L2 $27 \pm 4\%$ vs $38 \pm 3\%$; L3 $50 \pm 5\%$ vs $39 \pm 3\%$; L4 $51 \pm 8\%$ vs $51 \pm 4\%$).

Table 2 5-yr and 10-yr cumulative risks of intestinal and perianal complications in non-appendectomized and appendectomized patients according to disease location

| Location | | Non-appendectomized | | | | Appendectomized | | | |
|---------------------------|-------|---------------------|------------|------------|------------|---------------------|---------------------|------------|-----------|
| | | L1 | L2 | L3 | L4 | L1 | L2 | L3 | L4 |
| Number of patients | | 493 | 546 | 513 | 218 | 279 | 172 | 186 | 79 |
| Intestinal stricture | 5-yr | 23 ± 2 | 2 ± 1 | 9 ± 1 | 22 ± 3 | 30 ± 3 ^b | 3 ± 2 | 12 ± 3 | 29 ± 6 |
| | 10-yr | 34 ± 3 | 7 ± 1 | 17 ± 2 | 42 ± 5 | 46 ± 4 ^b | 6 ± 2 | 24 ± 4 | 42 ± 7 |
| Intestinal perforation | 5-yr | 30 ± 2 | 7 ± 1 | 12 ± 2 | 12 ± 2 | 27 ± 3 | 4 ± 2 ^a | 13 ± 3 | 10 ± 4 |
| | 10-yr | 42 ± 3 | 12 ± 2 | 25 ± 2 | 23 ± 4 | 37 ± 4 | 4 ± 2 ^a | 23 ± 4 | 29 ± 7 |
| Perianal fistulization | 5-yr | 14 ± 2 | 32 ± 2 | 31 ± 2 | 23 ± 3 | 11 ± 2 | 19 ± 3 ^b | 26 ± 4 | 15 ± 4 |
| | 10-yr | 18 ± 2 | 49 ± 3 | 41 ± 3 | 29 ± 4 | 17 ± 3 | 33 ± 5 ^b | 40 ± 5 | 21 ± 6 |

^b*P* < 0.001, ^a*P* < 0.05 vs appendectomized non-appendectomized patients (log rank). Other curves were not significantly different.

Table 3 Cumulative therapeutic needs in non-appendectomized and appendectomized patients according to disease location

| Location | Non-appendectomized | | | | Appendectomized | | | |
|---------------------------------|---------------------|------------|------------|------------|-----------------|------------|------------|-----------|
| | L1 | L2 | L3 | L4 | L1 | L2 | L3 | L4 |
| Number of patients | 493 | 546 | 513 | 218 | 279 | 172 | 186 | 79 |
| Oral or IV steroids | 396 (80) | 469 (86) | 462 (90) | 195 (89) | 228 (82) | 150 (87) | 173 (93) | 71 (90) |
| Enteral or parenteral nutrition | 53 (11) | 1 | 94 (18) | 48 (22) | 23 (8) | 0 | 35 (19) | 13 (16) |
| Azathioprine or methotrexate | 163 (33) | 305 (56) | 290 (57) | 132 (61) | 100 (36) | 83 (48) | 101 (54) | 48 (61) |
| Infliximab | 9 (2) | 73 (13) | 48 (9) | 19 (9) | 4 (1) | 11 (6) | 14 (8) | 5 (6) |
| Intestinal resection | 283 (57) | 173 (32) | 176 (34) | 79 (36) | 165 (59) | 42 (24) | 73 (39) | 30 (38) |

Numbers in parentheses are percentages. Comparison between non-appendectomized and appendectomized patients revealed no significant difference

Table 4 Comparison between two groups of patients included in prospective follow-up study

| | Non-appendectomized | Appendectomized |
|----------------------------|---------------------|-----------------|
| Number of patients | 591 | 591 |
| Number of females | 427 | 427 |
| Mean age at diagnosis (yr) | 31.8 ± 13.8 | 32.3 ± 13.4 |
| Mean age at inclusion (yr) | 34.0 ± 13.9 | 34.8 ± 13.7 |
| Diagnosis after 1987 (n) | 539 | 524 |
| Diagnosis after 1995 (n) | 351 | 357 |
| L1 location (n) | 202 | 204 |
| L2 location (n) | 162 | 160 |
| L3 location (n) | 156 | 156 |
| L4 location (n) | 71 | 71 |

No difference between the two groups was significant

Prospective study

The prospective study included 591 appendectomized patients and 591 non-appendectomized matched controls, both selected on the basis of not having had ileo-cecal resection at inclusion into follow-up. The two groups were well matched (Table 4). One hundred and forty-five appendectomized (25%) and 143 non-appendectomized patients (24%), respectively, had an ileo-cecal resection after inclusion. After censoring, the median length of follow-up for the two cohorts was 4 years and encompassed a total of 5478 patient-years. The rate of years with active disease was 50% in appendectomized patients (1 318 out of 2 637 patient-years) vs 51% in non-appendectomized patients

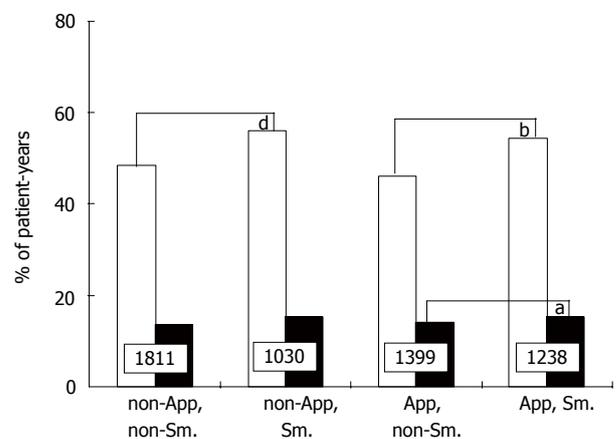


Figure 4 Percentages of patient-years with active disease (grey columns) and hospitalization (black columns), respectively, according to appendectomy and smoking status (App: appendectomized; Sm: smokers). Numbers indicate the number of patient-years in each group. ^a*P* < 0.05 vs App, non-Sm.; ^b*P* < 0.001 vs App, non-Sm.; ^d*P* < 0.001 vs non-App, non-Sm.

(1 455 out of 2 841 patient-years, NS). By contrast, in the same series of patients, non- or ex-smoking was associated with a decreased activity rate (1 521 out of 3 210 patient-years, 47%), compared to current smoking (1 252 out of 2 268, 55%; *P* < 0.001). Figure 4 gives the percentage of patient-years with active disease and hospitalization respectively, according to both smoking and appendectomy status. Previous appendectomy had no effect on year-by-year disease activity while smoking was significantly deleterious.

DISCUSSION

The present results show that previous appendectomy, while associated with some particularities in the location and behaviour of Crohn's disease, has no effect upon the course of the disease. Appendectomized patients are more prone to ileal disease and to formation of strictures, and less to distal colonic involvement and penetrating perianal disease. However, previous appendectomy does not change disease severity retrospectively assessed from therapeutic needs or prospectively assessed from year-by-year activity.

The role of appendectomy in Crohn's disease is difficult to assess because various biases may jeopardize the analysis. First, appendicitis may be the first manifestation of Crohn's disease. The appendix is frequently involved by Crohn's disease^[17], granulomatous appendicitis may in some cases reveal Crohn's disease^[18,19], and resection of a Crohn's disease appendix may be followed by a long period of remission^[20,21]. However, most pathological studies concluded that idiopathic granulomatous appendicitis is nosologically distinct from Crohn's disease^[22] and that most of these patients do not develop subsequent Crohn's disease. Moreover, when analyzing a cohort of Crohn's disease patients, Crohn's disease confined to the appendix was very unusual, observed in less than 1% of Crohn's disease patients^[23]. Although the pathology reports of the removed appendix were not available in our series, the data suggest that the great majority of our patients had appendectomy performed not for Crohn's disease. Moreover, the frequency of appendectomy in our series is similar to other series^[12] and not different from controls without Crohn's disease^[24,25]. Another difficulty is to accurately define in time the patient population in which the effect of appendectomy may be assessed. On one hand, inclusion of all appendectomized patients whatever the time span between appendectomy and the onset of Crohn's disease may lead to an overrepresentation of ileal disease cases, as an acute abdominal pain in the lower right quadrant may reveal ileal Crohn's disease. On the other hand, prolonging the time span between appendectomy and Crohn's disease may select cases of Crohn's disease occurring later in life and thus susceptible to share a lesser activity. Actually, in the present study, the comparison of patients subgroups defined by the duration of the interval between appendectomy and onset of Crohn's disease showed that only patients appendectomized within one year prior to Crohn's disease diagnosis clearly differed from other subgroups through a higher prevalence of ileal disease, as it could be expected (Figure 1). The characteristics of the subgroup of appendectomized patients one to five years prior to Crohn's disease diagnosis were intermediate between the two adjacent subgroups. This led us to define the cutoff at five years prior to appendectomy. Such a cutoff is in agreement with the data of Frisch *et al*^[6] who found that, five years after appendectomy, the relative risk of Crohn's disease was not further elevated. Finally, in the present study, selection bias were minimal, as we did not use a postal questionnaire, but included prospectively all consecutive patients seen over a 10-year period. The criteria used to assess the severity of the disease were objective, and were based on therapeutic needs. Both surgical and immunomodulator history are

accepted as useful indicators of the over-all severity of Crohn's disease because these interventions are both easy to document retrospectively, and their use and the effects of their use may be complementary^[14, 26]. Moreover, the prospective part of the study confirmed the results of the retrospective analysis.

Crohn's disease patients with prior appendectomy differed from non-appendectomized Crohn's disease patients in many aspects. There were more often females, smokers, of low-to-moderate socioeconomic status, and of Caucasian origin. In fact, these particularities might be more a consequence of appendectomy as such, than related to a particular Crohn's disease phenotype. Indeed, an epidemiological national survey in 1978-82 in France showed that appendectomy, but not appendicitis, was more frequent in females than in males^[24], and an association has been shown between appendectomy and passive or active smoking^[27]. Other epidemiological studies from UK have also reported an increased rate of incidental appendectomies in women^[28]. Similarly, the contrast between a higher proportion of Caucasian patients and a lower proportion of patients of high socio-economic status fits better with what is known about epidemiology of appendicitis than with IBD^[29,30]. In the Western world, appendectomy is performed more often in whites than in blacks^[31], whereas data from UK suggest that the incidence of IBD in immigrants is similar to that of Europeans^[32]. Changes in hygiene and water supply increased the risk of appendectomy^[33] which in the last four decades has preferentially concerned populations of low-to-moderate socioeconomic status, whereas patients with IBD seem to be better educated^[34]. We believe that the overrepresentation of ileal disease in appendectomized Crohn's disease patients may be at least in part a consequence of the high prevalence of smokers in this group, smoking being associated with ileal Crohn's disease and not colonic Crohn's disease^[35]. Alternative explanations of the more frequent ileal involvement in appendectomized patients would be a larger use of explorative procedures such as barium follow-through or colonoscopy because patients with previous appendectomy are more prone to small bowel obstruction^[36,37] and non specific abdominal pain^[38]. The occurrence of abdominal pain may give the opportunity to find out a paucisymptomatic ileitis which could have been ignored otherwise.

Comparisons of disease behaviour between appendectomized patients and controls revealed some subtle differences within each disease location group. The former patients were more exposed to stricture formation and less to penetrating anal disease. Actually, within the colon only group (L2 group), disease location tended to be more distal in non-appendectomized patients, perianal lesions were more frequent, and it could be expected that perianal perforating complications occurred more frequently. The increased prevalence of intestinal strictures in patients with prior appendectomy, although weak, seems more relevant. It may be hypothesized that the presence of intra-abdominal adhesences and/or vasculature modifications secondary to prior laparotomy has a role in stricture formation.

The most important and unequivocal result of the pres-

ent study is the lack of effect of prior appendectomy on the severity of Crohn's disease. We observed an increased risk of surgery in appendectomized patients, but multivariate analysis revealed that this was related to confounding factors. Riegler *et al*¹² have reported an increased risk of surgery in 41 Crohn's disease patients with previous appendectomy when compared to 88 non-appendectomized patients, but patients appendectomized within one year prior to diagnosis of Crohn's disease were included in the analysis. In the study by Andersson, compared to Crohn's disease controls, Crohn's disease patients with a history of perforated appendicitis had a higher incidence of intestinal resections, those with appendectomy for other diagnoses had a lower incidence, but the incidence for the whole group with prior appendectomy was similar to controls⁷. Radford-Smith *et al*³ have reported similar results in a retrospective analysis of 335 patients with Crohn's disease, of whom 36 had had prior appendectomy. Thus the effect of prior appendectomy on the risk of surgery seems nil or weak, or not related to appendectomy per se. Besides, therapeutic needs in terms of steroid and immunosuppressants requirements were very similar in appendectomized and non-appendectomized patients, whatever the disease location. Finally, the prospective follow-up of our appendectomized patients compared with controls matched for gender, age, and disease location, clearly demonstrated the lack of effect of prior appendectomy on the disease activity. This study was powered enough to support a firm conclusion, since concomitantly among the same patients, the deleterious effect of current smoking was highly significant.

In conclusion, this study shows that prior appendectomy is associated with a distinct phenotype of Crohn's disease. However these particularities may be attributable more to appendectomy than to Crohn's disease. Besides, in contrast to smoking, appendectomy has no effect on Crohn's disease severity. Taken together, these data indicate that appendectomy and Crohn's disease share common environmental or genetic characteristics whereas appendectomy per se does not exert any immune modulating effect. The response of IBD to the only environmental factors clearly documented so far, smoking and appendectomy, is quite different in UC and in Crohn's disease.

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Short- and medium-term reproducibility of gastric emptying of a solid meal determined by a low dose of ^{13}C -octanoic acid and nondispersive isotope-selective infrared spectrometry

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Abstract

AIM: To evaluate the reproducibility of a modified ^{13}C breath test-based measurement of solid phase gastric emptying (GE) within the frames of a simple-repeated measure study protocol.

METHODS: Twelve healthy subjects (6 females and 6 males, mean age 24.9 ± 0.7 years) were recruited to undergo three identical GE examinations. In six subjects the first two examinations were performed 2 d apart, and the third session was carried out at a median interval of 19.5 d (range 18 - 20 d) from the second one. In another six subjects the first two measurements were taken 20 d apart (median, range: 17-23 d), whereas the third session took place 2 d after the second one. Probes of expiratory air collected before and during six hours after intake of a solid meal (378 kcal) labelled with $75 \mu\text{L}$ (68 mg) ^{13}C -octanoic acid, were measured for $^{13}\text{CO}_2$ enrichment with the nondispersive isotope-selective infrared spectrometry NDIRS apparatus.

RESULTS: Taking coefficients of variation for paired examinations into account, the short-term reproducibility of the GE measurement was slightly but not significantly better than the medium-term one: 7.7% and 11.2% for the lag phase (T-Lag), 7.3% and 10.9% for the gastric half emptying time ($T_{1/2}$). The least differences in GE parameters detectable at $P=0.05$ level in the 12 paired examinations were 9.6 and 15.6 min for T-Lag, 11.6 and 19.7 min for $T_{1/2}$ by a two-day or two to three-week time

gap, respectively.

CONCLUSION: The low-cost modification of the breath test involving a lower dose of ^{13}C -octanoic acid and NDIRS, renders good short- and medium-term reproducibility, as well as sensitivity of the measurement of gastric emptying of solids.

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Key words: ^{13}C breath test; Gastric emptying; Nondispersive isotope-selective infrared spectrometry; ^{13}C -octanoic acid; Reproducibility

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INTRODUCTION

Since its first description and validation in 1993^[1], ^{13}C -octanoic acid breath test (^{13}C -OABT) has dug its way towards the top of the list of the most widely used methods for the measurement of gastric emptying of solids. For example, recently published epidemiologically-oriented studies addressing the features of gastric emptying kinetics in large cohorts of patients involving hundreds of subjects suffering from functional dyspepsia, have been performed with ^{13}C -OABT^[2-4]. Also the introduction of less expensive and technically demanding apparatus, based on the non-dispersive isotope-selective infrared spectrometry (NDIRS), is an undeniable milestone which has opened the gate for a widespread use of ^{13}C breath tests^[5-7], the ^{13}C -OABT making no exception in this respect^[8-13].

Reproducibility is a vital feature of any measurement or diagnostic method used in medical practice. Few data are available on the repeatability of the ^{13}C -OABT in humans, and reports addressing this subject appear unsatisfactory^[14,18]. Moreover, no previous study has reported reproducibility of the NDIRS variety of the ^{13}C -OABT. The aim of this study was to establish the short- and medium-term repro-

ducibility of the ^{13}C -OABT with NDIRS and a reduced dose of the ^{13}C substrate.

MATERIALS AND METHODS

Subjects

Twelve healthy subjects (6 females and 6 males) were recruited to participate in this study, their mean age was 24.9 ± 0.7 years, and the average body mass index amounted to $22.37 \pm 1.06 \text{ kg/m}^2$.

During a screening interview, the participants declared themselves to be in full health according to the World Health Organisation criteria. Exclusion criteria comprised a history of surgery affecting the digestive tract anatomy with the exception of appendectomy, current use of any drugs which might affect gastrointestinal motility, and pregnancy. The recruitment procedure involved performance of a standard breath test with ^{13}C -urea, so that exclusively subjects with a negative test result for *Helicobacter pylori* infection were admitted. Except for three persons, all the participants were non-smokers. The study was conducted in accordance with the Helsinki Declaration, and each volunteer gave a written consent after getting information as to the aim, protocol and methodology of the study. During the introductory interview, the subjects were committed not to eat any food of naturally increased ^{13}C content, such as products made of maize, cane sugar, pineapple, kiwi fruit for 48 h preceding the examination.

Experimental protocol

Each subject underwent three sessions of gastric emptying measurement held on separate days. In six subjects the first two examinations were performed 2 d apart, and the third session was carried out at a median interval of 19.5 d (range 18-20 d) from the second one. In another six subjects, the first two gastric emptying measurements were taken 20 d apart (median, range 17-23 d), whereas the third session took place 2 d after the second one. The assignment of the order of intervals separating the sessions (short-long or long-short) was randomized. Ultimately the short-term reproducibility assessment involved performance of 12 pairs of gastric emptying examinations separated by a two-day break, whereas twelve pairs of examinations accomplished at a median interval of 21.5 d (range 17-23 d) were dedicated for the evaluation of the medium-term reproducibility.

The research was performed on volunteers who reported themselves to the laboratory in the morning, after a 12-h overnight fasting and abstaining from cigarette smoking (if applicable). At the beginning, a basal fasted probe of the exhaled air was put into an aluminium-covered plastic bag of about 1 L capacity (Fisher Analysen Instrumente GmbH, Germany). At the time point of "0", the subjects were given a solid test meal, a pancake made of two eggs, 30 g wheat flour and 0.1 g baking powder, which was additionally smeared with 50 g of strawberry jam before serving. The total energy content of the meal was 1574 kJ (378 kcal) and it contained 15.5 g proteins, 16.8 g fat, and 43.0 g carbohydrates. During the preparation procedure of the pancake, the two-egg yolks were temporarily separated from the egg whites and thoroughly mixed with 75 μL (68

mg) of ^{13}C -octanoic acid (INC610P, lot #T012A-L3241, Euriso-Top, France) which was instilled with a precision digital micropipette (Calibra 822-20/200, Socorex, Switzerland). Thereafter the yolks were added to and stirred with the remaining ingredients with an electric mixer. Finally the dough was transferred into a pan and fried to firm consistency with addition of 5 mL of sunflower oil. The time needed to consume the test meal did not exceed 10 min. No drink was served with the meal because the study intended to assess strictly the reproducibility of the solid phase gastric emptying.

Reckoning the passage of time from the defined "0" point, 26 probes of the expiratory air were collected post-prandially every 10 min during the first hour, and then every 15 min for an additional 5 h. After ingestion of the meal, the subjects were asked not to take any additional food or drink for 6 h. They were enabled to rest sitting in a comfortably furnished room and allowed to watch video films.

Measurement of $^{13}\text{CO}_2$ and derivation of gastric emptying parameters

Concentrations of $^{13}\text{CO}_2$ in the probes of the exhaled air were measured with the NDIRS apparatus (*IRIS*, Wagner Analysen Technik Vertriebs GmbH, Germany; a model equipped with 16 ports for simultaneous mounting of bags with air samples was used). Repeatability of the $^{13}\text{CO}_2$ measurement with the NDIRS technique was checked by double measurements performed on 96 probes of the exhaled air originating from 3 randomly selected gastric emptying examinations. The procedure was performed in such a way that the second measurement was taken instantly without removing the bags containing the probes from the apparatus.

The determined $^{13}\text{CO}_2$ content within the total pool of the exhaled CO_2 was expressed in $\delta\text{‰}$ PDB units, i.e. relative to the international standard - the calcium carbonate of the fossil Belemnite of the cretaceous Pee Dee formation in South Carolina, USA (zero $\delta\text{‰}$ PDB corresponds to 1.12372% ^{13}C atoms within CaCO_3). The obtained $\delta\text{‰}$ PDB data were exported to an ASCII file for a subsequent analysis with a user-created Excel spreadsheet.

The changes in $^{13}\text{CO}_2$ concentration were recalculated to net increments expressed in ‰ DOB (*delta over baseline*) units according to the formula:

$$\text{DOB}_i = \delta\text{‰ PDB}_i - \delta\text{‰ PDB}_0$$

where i stands for the probe number, and the 0 index pertains to the basal probe of the expiratory air.

The momentary ^{13}C recovery at time i , $\text{D}^0\text{‰}_{i-13}\text{C}$, expressed in percent age of administered dose per hour (‰ dose per hour), was computed according to the equation:

$$\text{D}^0\text{‰}_{i-13}\text{C} = 100 \cdot (\text{DOB}_i / 1000 \cdot 0.0112372 \cdot \text{TCO}_2) / \text{dose}$$

where TCO_2 = total expiratory CO_2 production in mmol/h and dose = amount in mmol of ^{13}C -octanoic acid given to the subjects. The TCO_2 was derived by multiplication by the rate of 300 mmol/m^2 per hour by the body surface area computed according to the formula by Haycock *et al*^[19].

The ^{13}C recovery within a given period, C_i-^{13}C , ex-

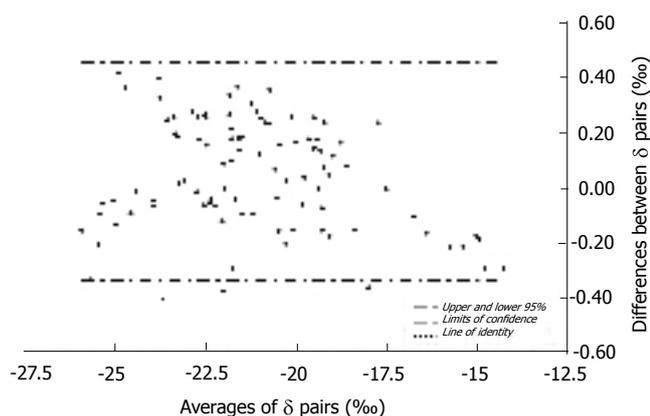


Figure 1 Repeatability of the measurement of ¹³CO₂ enrichment (δ) in expiratory air after intake of a solid meal labeled with 75 μ L (68 mg) ¹³C-octanoic acid. Ninety-six pairs of δ measurements taken from three randomly selected examinations were considered.

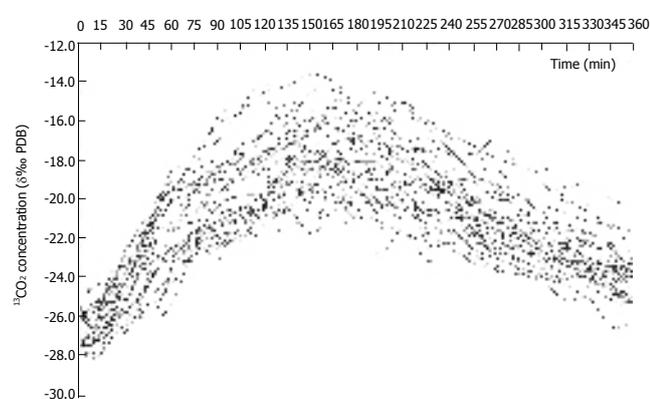


Figure 2 Individual curves reflecting the increment in ¹³CO₂ enrichment in exhaled air after intake of a 378 kcal solid meal containing 75 μ L (68 mg) ¹³C-octanoic acid (the 36 curves were obtained in 12 healthy subjects at three separate days).

pressed in percent age of dose, was computed following the trapezoid rule:

$$C_i \text{ } ^{13}\text{C} = 0.5 \cdot (\text{DOB}_{i-1} + \text{DOB}_i) / 1000 \cdot \Delta t \cdot 0.0112372 \cdot \text{TCO}_2 / \text{dose}$$

where Δt = the time span between consecutive DOB measurements

The cumulative ¹³C recovery T_i - ¹³C in percent age of administered dose was then derived from a stepwise summation of C_i - ¹³C for i within the domain of 1 - 26.

Using algorithms of non-linear regression implemented in the Statistica 6.1 software^[20], the curves of momentary ¹³C recovery were fitted to the function:

$$D^{\%}_{oi} \text{ } ^{13}\text{C} = a \cdot e^{-ct}$$

where t stands for time, and a , b , c are parameters of the function

which enables computation of three gastric emptying parameters^[11,21]: the lag phase, $T\text{-Lag} = b/c$; the gastric half emptying time, $T_{1/2} = \text{Gamma inv.}(0.5; b+1; 1/c)$; and the gastric emptying coefficient, $\text{GEC} = \ln(a)$

Statistical analysis

A null hypothesis, assuming a zero difference between repeated measurements, was checked with the paired

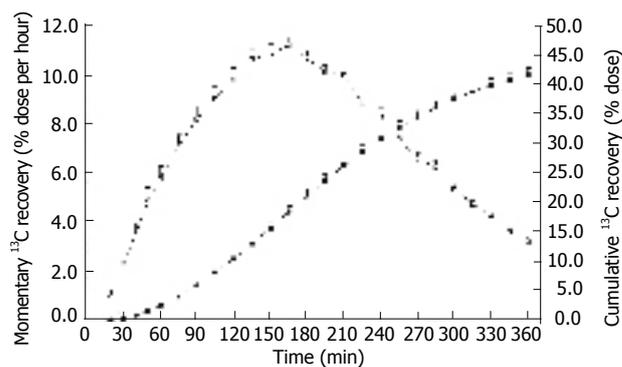


Figure 3 Momentary (empty squares) and cumulative (filled squares) ¹³C recovery in exhaled air after ingestion of a 378 kcal solid meal containing 75 μ L (68 mg) ¹³C-octanoic acid. The data are grand means with standard errors (bars) calculated on average values obtained in 12 healthy volunteers during three examinations carried out on separate days.

Student's t test. The reproducibility of the gastric emptying parameters was expressed in terms of the coefficient of variation for paired examinations, CV_p ^[22,23]. Moreover Bland and Altman statistics was applied in order to calculate the repeatability coefficient, RC ^[24,25]. Finally, $\Delta_{0.05}$ - the least statistically significant difference detectable at $P=0.05$ level (two-tailed) was calculated for each gastric emptying parameter^[26]. Results were presented as mean \pm SE. All statistical analyses were performed with the Statistica 6.1 software, licence # adbp409a903816ar^[20].

RESULTS

Repeatability of NDIRS measurement of ¹³CO₂ enrichment in exhaled air

Bland and Altman statistics performed on the paired measurements of the ¹³CO₂ content within 96 samples of the expiratory air yielded a repeatability coefficient of 0.40‰, and the mean difference between the repeated measurements amounted to 0.07‰ (95% confidence interval: 0.34 - 0.46‰) (Figure 1). If the data pertaining to the ¹³CO₂ content within the expiratory air were expressed in terms of the delta over baseline values, the respective repeatability coefficient amounted to 0.62‰, with a mean difference of 0.28‰ between repeated measurements (95% confidence interval: 0.34 - 0.90‰).

Reproducibility of parameters characterizing gastric emptying kinetics

Ingestion of the solid test meal labeled with 75 μ L of ¹³C-octanoic acid brought about an expected increase of the ¹³CO₂ concentration in the exhaled air (Figure 2). The maximum net increment in the ¹³CO₂ concentration amounted to 9.1‰ (mean, 95% confidence intervals 5.3‰ to 12.9‰) during the 36 gastric emptying examinations. The maximum momentary ¹³C recovery in the exhaled air was observed at 165 min (mean, 95% confidence intervals 112 min to 219 min) postprandially, and amounted on average to 11.50 ± 0.36 % dose per hour (Figure 3). During the whole 6 h observation period 41.80 ± 1.13 % of the administered tracer was eliminated in the form of ¹³CO₂ within the expiratory air (Figure 3).

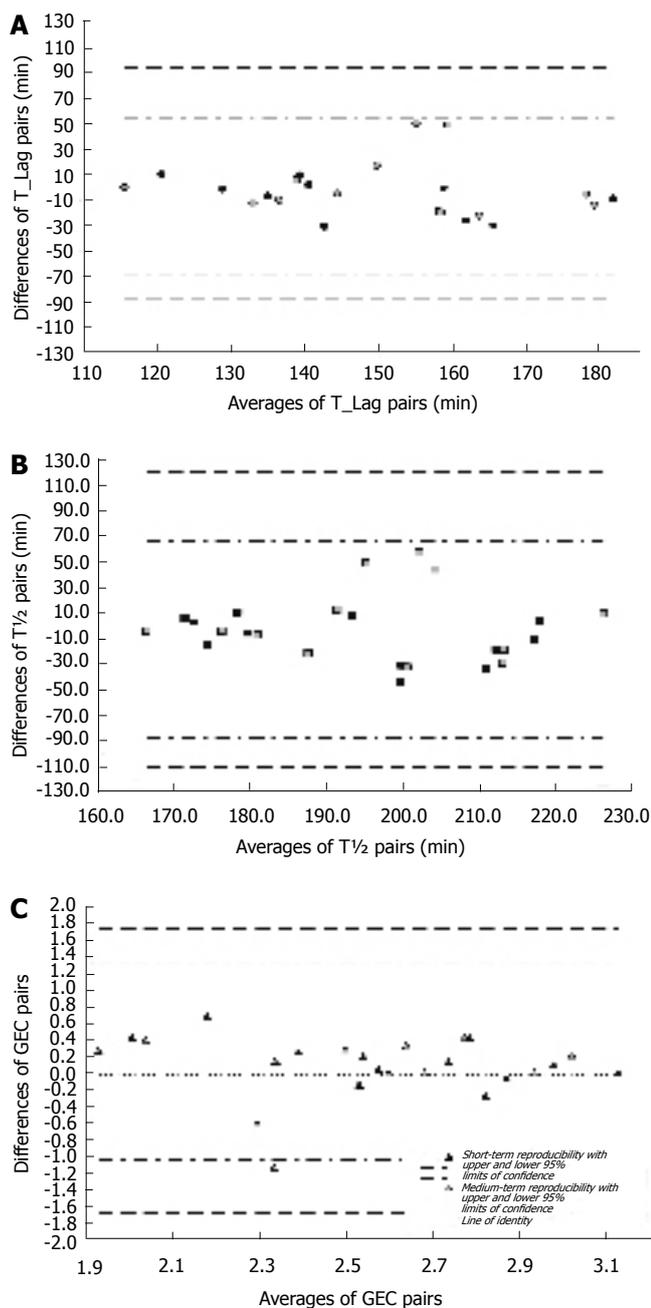


Figure 4 Bland and Altman statistics (plot of differences between pairs vs their names) of the short- (open symbols) and medium-term (filled symbols) reproducibility of the measurement of the lag phase (T-Lag, panel A), the gastric half emptying time (T_{1/2}, panel B), and the gastric emptying coefficient (GEC, panel C) of the solid phase gastric emptying with a breath test using 75 μ L (68 mg) ¹³C-octanoic acid and the isotope-selective nondispersive infrared spectrometry. On each panel the respective borders of the 95% confidence intervals are plotted - cf. the legend of the GEC graph, panel C.

A graphical depiction of the Bland and Altman statistics pertaining to the gastric emptying parameters is given in Figure 4, and a comprehensive statistical description of the reproducibility data is provided in Table 1. In no instance the null hypothesis of a zero difference between the repeated measurements had to be discarded. According to the data assembled in Table I, greater values of RC and CV_p characterized the medium-term compared to the short-term reproducibility. A comparison of the absolute values of the differences between the paired measurements implied rejection of a hypothesis that reproducibility of the

gastric emptying was significantly worse when the examinations were separated by an extended time gap.

DISCUSSION

The novelty of the results furnished by the current study consists in: for the first time the reproducibility of the ¹³C-OABT was evaluated with the NDIRS, a reduced dose of the ¹³C-octanoic acid was applied for the test, the test meal was administered without allowance of any intake of liquids so that pure repeatability of the solid phase gastric emptying could be discerned, and a distinguishment was made between the short- and medium-term reproducibility.

Typically the ¹³C-OABT is performed with 100 μ L of ¹³C-octanoic acid which is equivalent to 91 mg^[2-5,8-12,14,16-18]. It was reported that a lower dose - 50 mg ¹³C-octanoic acid can be used to examine the solid phase gastric emptying^[21]. The NDIRS apparatus coped well with the 68 mg (75 μ L) amount of ¹³C-octanoic acid applied for labelling the test meal in the current study. This contention is supported by the finding of a low value of the repeatability coefficient (0.40%) and a narrow 95% confidence interval of the differences between paired measurements of ¹³CO₂ enrichment (0.34 - 0.46‰) derived from a large number of breath samples covering a typical range of ¹³CO₂ concentration encountered throughout the 6 h of the ¹³C-OABT. Since baseline fluctuation of ¹³CO₂ concentration in breath is expected to approach a standard deviation of 0.72‰^[27], our results clearly indicate that the measurement error involved in the NDIRS technique is less than the natural baseline fluctuation of ¹³CO₂ concentration in breath. The finding quoted is in concordance with our previously published results^[6,7,28].

While performing the ¹³C-OABT, we generously allowed for a frequent sampling of the expiratory air (a total of 27 samples were collected throughout a single examination including the basal specimen) over a long observation period of 6 h. This approach was substantiated by findings of other authors clearly stating that too short a collection period and/or an infrequent sampling of the expiratory air may lead to erroneous estimation of the parameters of gastric emptying with the ¹³C-OABT^[16,17,29]. According to Choi *et al*^[16,17], duration of the ¹³C-OABT obligatory should be extended to six hours, since largely overestimated values of T-Lag and T_{1/2} would be derived from the data truncated to four hours.

Three parameters are originally proposed as quantitative descriptors of the gastric emptying kinetics examined with the ¹³C-OABT, namely: T-Lag, T_{1/2}, and GEC^[1,21]. Nowadays researchers are increasingly inclined to use the T_{1/2} only when reporting on their results with the ¹³C-OABT. This apparently minimalistic approach has quite firm grounds. The shortcomings of GEC consist in the fact that its estimation is not independent of the endogenous CO₂ production, and in the case of fast gastric evacuation patterns this parameter may underestimate gastric emptying^[1]. T-Lag of the ¹³C-OABT is originally conceived to reflect the duration of the first phase of gastric evacuation, while the solid meal is ground to particles of 1-2 mm in diameter before the actual emptying can start. This assumption has not found any convincing corroboration in vali-

Table 1 Reproducibility of solid gastric emptying measurement with breath test at a 68 mg (75 µL) dose of ¹³C-Octanoic acid and isotope-selective nondispersive infrared spectrometry

| Observed values ¹ | T-Lag, lag phase 149.7 ± 4.6 min | | T½, gastric half emptying time 195.6 ± 4.2 min | | GEC, gastric emptying coefficient 2.56 ± 0.09 | |
|------------------------------|-------------------------------------|-------------|---|-------------|--|-------------|
| | Short-term | Medium-term | Short-term | Medium-term | Short-term | Medium-term |
| RC (min) | 31.6 | 46.7 | 39.1 | 59.1 | 0.61 | 0.87 |
| CVp (%) | 7.7 | 11.2 | 7.3 | 10.9 | 8.3 | 12.5 |
| △ 0.05 (min) | 9.6 | 15.6 | 11.6 | 19.7 | 0.17 | 0.29 |

Short-term reproducibility involved twelve paired examinations performed 2 d apart, whereas medium-term reproducibility involved twelve paired examinations at a median interval of 21.5 d (range 17 - 23 d)

RC = repeatability coefficient, CVp = coefficient of variation for paired examinations, △ 0.05 = the least statistically significant difference detectable at $P=0.05$ level, two-tailed

¹ These data are grand means calculated on average values obtained during three examinations carried out on separate days

dation studies of the ¹³C-OABT against the scintigraphic determination of gastric emptying, which is the so called 'golden standard' in the field of gastro-emptology^[16,17]. Quite recently it was even demonstrated by Doppler ultrasonography that transpyloric flow starts already during ingestion of a ¹³C-labelled solid meal, which results in a detectable excretion of ¹³CO₂^[30]. It has been shown that trituration of the solid meal can lead to a pronounced shortening of this parameter^[31]. Nevertheless, T_Lag will be more and more willingly replaced by the term 'time to reach the maximum of the momentary ¹³CO₂ excretion' which is the revised representation of what the T-Lag derived mathematically from the function fitted to a ¹³CO₂ excretion curve actually is^[18,9,21].

In the present study, we controlled rigorously and kept constant a number of factors which might affect reproducibility of the ¹³C-OABT, namely meal composition, time of the day when the gastric emptying measurement was taken, posture of the subjects during the test. Ingestion of the test meal took place irrespective of actual phase of the duodenal migrating motor complex (MMC), because monitoring of the duodenal motor activity would require insertion of a manometric tube into the lumen of the duodenum - an invasive manoeuvre which might interfere with physiological conditions assumed for the ¹³C-OABT performance; observation of a particular phase of the duodenal MMC could not ameliorate the reproducibility of the scintigraphic measurement of the gastric emptying of the solid phase of a meal^[32]. Homogeneity was the important feature of the test meal we applied, that is the pancake was uniformly labelled with ¹³C-octanoic acid and eaten without addition of any liquid to assure the sole assessment of the reproducibility of the solid phase gastric emptying^[33].

Because there are no other reports on the ¹³C-OABT performed with the NDIRS, our data can be compared only to the results obtained by other authors applying the isotope ratio mass spectrometry (IRMS). Choi *et al*^[17] reported that the reproducibility of T-Lag and T½ is 14% and 15% respectively. In the study by Delbende *et al*^[18] the pertinent RC and CV_p amounted to 35.5 min and 14.3%, respectively. The reproducibility results of those two studies look very much alike indeed. The results of the present study indicated that the reproducibility of gastric emptying of solids determined by ¹³C-OABT and the NDIRS was not worse than that by IRMS. Moreover, both the reproducibility and sensitivity (as assessed by the magnitude

of the least detectable differences in the gastric emptying parameters) were preserved when the examinations were separated by a two- three-week time gap. Finally, it should be emphasized that the results obtained by others^[17,18] and coming from our current study clearly demonstrate that the reproducibility of the measurement of gastric emptying of solids by the ¹³C-OABT is equivalent to the intra-subject variability of the solid-phase gastric emptying observed with gamma scintigraphy^[22,23]. This is an important finding due to the inherently indirect nature of ¹³C-OABT. The reproducibility of ¹³C-OABT would be affected by more factors than a scintigraphic GE measurement, namely the intestinal absorption of the ¹³C-octanoic acid, its oxidation to ¹³CO₂ within the liver, as well as kinetics of the subsequent transfer of ¹³CO₂ to the expiratory air.

In conclusion, the low-cost modification of the breath test involving a lower dose of ¹³C-octanoic acid and NDIRS, renders a good short- and medium-term reproducibility, as well as sensitivity of the measurement of gastric emptying of a solid meal.

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RAPID COMMUNICATION

Higher platelet P-selectin in male patients with inflammatory bowel disease compared to healthy males

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Abstract

AIM: To observe if the total amount of platelet P-selectin (tP-selectin) in patients with inflammatory bowel disease (IBD) was related to disease entity or activity, 5-aminosalicylic acid (5-ASA) medication or gender.

METHODS: tP-selectin was measured by immunoassay in seventeen IBD patients and twelve healthy controls.

RESULTS: Compared to controls, there was no difference of tP-selectin in patients related to disease entity or activity and 5-ASA medication. When the groups were split according to gender the male patient group showed higher levels of tP-selectin compared to male controls (153 ng/mL vs 94 ng/mL, $P < 0.05$).

CONCLUSION: Increased tP-selectin levels may alter the inflammatory response and susceptibility to thromboembolic disease. As previously shown with soluble P-selectin, tP-selectin shows gender dependent differences important to consider in future studies.

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Key words: Platelet; P-selectin; Inflammatory bowel disease; 5-aminosalicylic acid; Gender

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INTRODUCTION

Upon platelet activation, intracellular P-selectin is expressed on the membrane surface and released from the platelets^[1]. In addition to P-selectin, platelets release various inflammatory mediators that regulate both hemostasis and inflammation^[2].

Ulcerative colitis (UC) and Crohn's disease (CD) are disorders of unknown aetiology, jointly referred to as inflammatory bowel disease (IBD). Current research suggests platelet dysfunction as a contributor to the disease since increased risk of thromboembolic disease and abnormal platelet activity have been found. Increased surface expression of platelet P-selectin in patients with IBD did not appear to reflect disease activity^[3,4], whereas soluble P-selectin in plasma recently was suggested to be parallel to the severity of UC^[5]. Using a new technique to quantify the total amount of P-selectin in platelets, patients with hypertension have been reported to have higher levels of platelet P-selectin^[6]. Our aim of this study was to observe if total amount of platelet P-selectin (tP-selectin) differs between IBD patients and healthy controls and if there is a difference regarding disease entity, disease activity, 5-aminosalicylic acid (5-ASA) medication and gender.

MATERIALS AND METHODS

Controls and patients with intake of anti-platelet drugs or steroids two weeks prior to sample collection were excluded. Seven female and five male healthy blood-donors served as a controls (mean age 44.9, range 25-65 years). The patient group consisted of six female and eleven male IBD patients (mean age 44.4, range 19-69 years). Diagnosis was verified with histological examination (biopsy and colonoscopy); twelve patients had UC and five patients had CD. Patients were sub-grouped according to disease activity, 5-ASA medication and gender. In UC eight patients were in relapse and in CD all patients were in remission. Ten patients had 5-ASA medication.

Isolated platelets (IP) were prepared as previously described^[7]. Frozen IP samples were thawed and incubated with 0.5% Triton X-100 (Sigma Chemical Co., St. Louis, MO, USA) at 37 °C for 1 h. Thereafter, the permeabilised IP samples were gently mixed prior to measurement of tP-selectin levels according to standard procedures using an immunoassay from R&D Systems (Abingdon, Oxfordshire, UK).

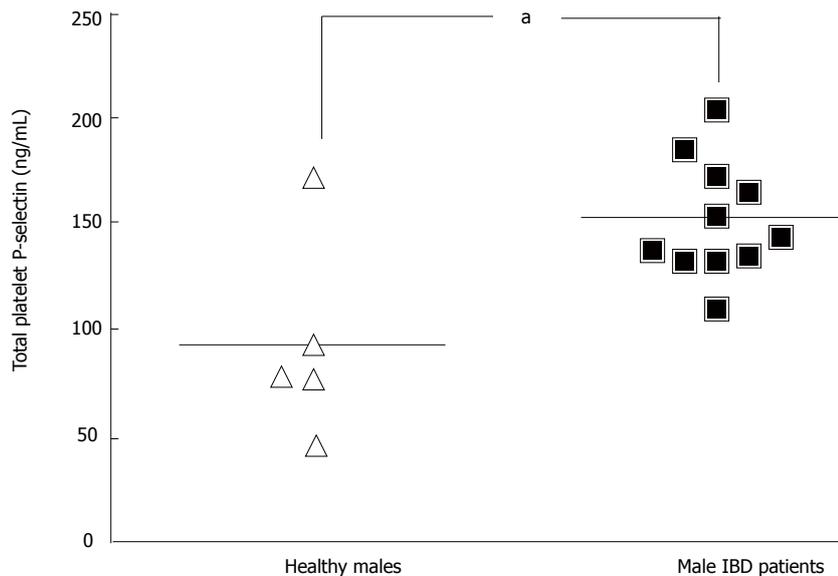


Figure 1 Total P-selectin in permeabilised platelets from 5 healthy male subjects and 11 male patients with inflammatory bowel disease. Horizontal lines represents the mean value. ^a $P < 0.05$ vs unpaired *t* test with Welch's correction.

Statistical analysis

All values are presented as mean \pm standard error of the mean (SE). Statistical analyses were calculated by unpaired *t* test with Welch's correction for samples with different variances. Differences were considered significant when $P < 0.05$.

RESULTS

All values are in ng/mL. There was no significant difference of tP-selectin between controls, 133 ± 20.1 ($n = 12$), and patients, 154 ± 8.1 ($n = 17$). When samples were sub-grouped according to gender the male patients showed significant higher levels compared to the male controls; 153 ± 8.2 and 94.1 ± 21.1 (Figure 1). In the sub-grouped male group the mean and range of age were 46.8 and 32-55 years in the control group and 46.4 and 24-65 years in the male patient group, denoting that age is not the cause of the different levels. Both the female controls and the female patients tended to have higher levels compared to male controls, although insignificantly. There were no significant differences between the female controls and female patients; 161 ± 27.7 ($n = 7$) and 156 ± 18.7 ($n = 6$). The levels of tP-selectin in UC compared to CD were 151 ± 9.6 ($n = 12$) and 163 ± 16.3 ($n = 5$). Values regarding disease activity showed following results; remission, 157 ± 11.2 ($n = 9$) and relapse, 152 ± 11.7 ($n = 8$). None of the groups showed significant difference in comparison with the controls. The levels in male patients in remission compared to male patients in relapse were; 155 ± 13.7 ($n = 6$) and 152 ± 9.2 ($n = 5$). Patients with 5-ASA compared to patients without 5-ASA had tP-selectin levels of 152 ± 9.1 ($n = 10$) and 159 ± 15.6 ($n = 7$), respectively. There was no significant difference between the male patients with 5-ASA, 151 ± 8.4 ($n = 6$) compared to male patients without 5-ASA, 156 ± 16.0 ($n = 5$).

DISCUSSION

Plasma levels of soluble P-selectin has recently been

suggested to relate to the severity of UC^[5] and increased tP-selectin has been found in hypertensive patients^[6]. Our previous study showed that patients with IBD in remission had higher basal platelet P-selectin surface expression^[4]. To investigate if this was related to higher platelet P-selectin content we analysed tP-selectin in patients with IBD. There was no difference of tP-selectin in patients taken as one group compared to controls, irrespectively of disease entity or activity and 5-ASA medication. When the groups were split according to gender the male patient group showed significantly higher levels compared to male controls. Both healthy and patient females showed greater variance compared to the male groups. This may be caused by a hormonal influence on tP-selectin, since sP-selectin fluctuates during the menstrual cycle^[8] and age (pre- and post-menopausal). Other sex-based differences that influence the disease activity in IBD have recently been reported^[9]. In the present study, male patients had higher tP-selectin levels regardless of disease entity, disease activity and 5-ASA medication. Taken together with our previous findings^[4], we suggest that male IBD patients have higher levels of tP-selectin and exhibit higher basal platelet P-selectin expression when in remission. This could have an exaggerated influence on the inflammatory response and susceptibility to thromboembolic disease.

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Does endothelium agree with the concept of idiopathic hepatic vessel thrombosis?

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tion and managed differently.

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Abstract

AIM: To investigate the major steps of thrombogenesis and to identify the differences in these steps between idiopathic patient group and control group.

METHODS: Fibrinogenesis was studied by measuring the activated factor VII, total and free levels of tissue factor pathway inhibitor (TFPI). The fibrinolysis step was investigated by determining the global fibrinolytic capacity. The endothelial function was assessed by measuring the levels of soluble adhesion molecules, namely soluble intercellular adhesion molecule 1 (sICAM-1), soluble vascular cell adhesion molecule 1 (sVCAM-1) and soluble E-selectin molecule. The exclusion criteria from "idiopathic" patient group were abdominal surgery, pregnancy, use of oral contraceptives, anti-phospholipid syndrome, Behçet's disease, cancer, myeloproliferative diseases. The congenital factors like mutations of factor-V Leiden and prothrombin, deficiencies of proteins C and S, antithrombin, hyperhomocysteinemia and hyperfibrinogenemia were ruled out. The total number of patients was reduced from 96 to 9 (7 with portal vein thrombosis, 2 Budd Chiari syndrome) by exclusion criteria.

RESULTS: The levels of adhesion molecules sICAM-1, sVCAM-1, free TFPI levels and global fibrinolytic capacity were significantly different ($P < 0.05$) in the patient group indicating an endothelial dysfunction and a lower fibrinolytic activity.

CONCLUSION: These results show that this patient group should be tested by means of endothelial dysfunction

INTRODUCTION

Thrombosis in the hepatic vascular system (hepatic veins and portal veins) is an established risk factor for both morbidity and mortality due to inevitable consequences of disturbed liver vascular physiology and anatomy. The primary mechanism resulting in this condition may be clinically evident (ie, a mass-forming lesion adjacent to a major vessel resulting in compression and occlusion, which is called a "secondary" thrombosis) and by definition these patients are treated with therapeutic techniques to eliminate the cause. On the other hand, in some patients there may be a thrombophilic condition resulting in a "primary" thrombosis which usually is the case in this patient population. The clinical importance of presence or absence of a thrombophilic condition lies in the fact that the presence of a thrombophilic condition increases the mortality by a factor of 4^[1].

Studies investigating the etiology of thrombosis in hepatic vasculature indicate that the most commonly-found predisposing thrombophilic conditions are deficiencies of proteins C and S as well as antithrombin (AT), mutations of factor V Leiden and prothrombin followed by myeloproliferative diseases and other less commonly-observed disorders including Behçet's disease^[2-6]. On the other hand, there seems to be a patient population in whom there is not any obvious thrombophilic condition that can be found utilizing routine techniques but mortality risk increases still in the high risk group due to undiagnosed thrombophilic condition. This unique population is called "idiopathic" thrombosis group and their contribution to the main patient population is about

Table 1 Applied exclusion criteria

| Exclusion criteria |
|--|
| Acute thrombosis of portal or hepatic veins |
| Any ultrasonographic (including Doppler studies) finding compatible with mass occupying lesion in liver |
| Positivity of viral hepatitis markers |
| Any finding in the liver biopsy suggesting parenchymal liver disease (including autoimmune liver diseases and metabolic liver diseases) |
| Acquired hematological abnormality leading to thrombophilia (myeloproliferative diseases, paroxysmal nocturnal hemoglobinuria, history of use of oral contraceptive or estrogen replacement treatment, anti-phospholipid syndrome, history of cancer, history of hepatobiliary surgery, history of pancreatitis) |
| Genetic hematological abnormality leading to thrombophilia (mutation of factor V Leiden, mutation of prothrombin, deficiencies in proteins C and S or antithrombin, hyperfibrinogenemia, hyperhomocysteinemia) |
| Severe and widespread atherosclerosis, uncontrolled or newly diagnosed malignancy elsewhere, systemic inflammatory diseases, history of recent operation |

7%-22%^[7,8]. In the current knowledge, hematological changes of this remaining “idiopathic” group is unclear, hence giving opportunity to investigate this patient population with advanced markers of thrombosis formation.

According to the current concept of pathologic thrombus formation, a thrombus is the result of convergence of multiple factors which may be either genetic or acquired. Therefore, it seems crucial to clarify the underlying possible pathogenetic factors or predisposing conditions in the “idiopathic” patient population in terms of tests not routinely used for the investigation of thrombogenesis.

In our study, we aimed to investigate the general characteristics of patients with idiopathic portal or hepatic vein thrombosis, to compare the “idiopathic” patient group with healthy adults in terms of major steps of thrombogenesis and fibrinolytic potentials, and to reconcile the “thrombophilia theory” in etiology of idiopathic portal hypertension with idiopathic portal vein thrombosis. The advanced tests that we used to investigate possible thrombophilia included activated factor VII (FVIIa) which is the most potent initiator of thrombin formation via tissue factor and its potent controller called tissue factor pathway inhibitor (TFPI; total and free fractions), adhesion molecules, namely soluble vascular cell adhesion molecule-1 (sVCAM-1), soluble intercellular adhesion molecule-1 (sICAM-1) and soluble E-selectin molecule as markers of endothelial dysfunction and global fibrinolytic capacity (GFC) to assess the steps of fibrinolysis.

This study was designed to investigate the endothelial dysfunction, steps of fibrinogenesis and fibrinolytic capacity in a patient group named as “idiopathic” after use of current techniques to investigate thrombophilia.

MATERIALS AND METHODS

Patient and control groups

All the patients diagnosed to have chronic portal vein or hepatic thrombosis with any appropriate diagnostic technique (Doppler-ultrasound, conventional angiography,

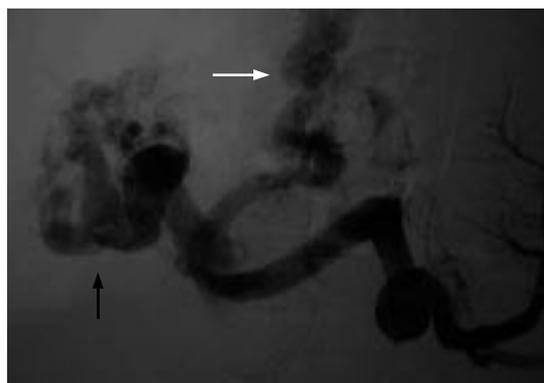


Figure 1 Splenoportal angiography showing cavernous transformation (bold arrow), esophageal varices (white arrow) indicating severe portal hypertension.

computerized tomographic angiography or magnetic resonance angiography) between January 2000 and December 2003 were evaluated for possible inclusion to the study. One splenoportal angiography of our patient group showed portal vein cavernous transformation (Figure 1). The exclusion criteria (as shown in Table 1) were applied to eliminate chronic parenchymal liver diseases including cirrhosis, mass occupying lesion around great vessels and any genetic or acquired abnormality leading to thrombophilia.

All the patients underwent a series of evaluation comprised of viral markers of hepatitis, screening tests of metabolic liver diseases (Wilson’s disease, hemochromatosis), liver biopsy, red blood cell count, full biochemical tests, reticulocyte counts and peripheral blood smear in order to rule out silent liver diseases or myeloproliferative blood diseases. Any increased hematocrit more than 50% indicated a scintigraphic total red cell mass count, whereas any abnormality in reticulocyte count or any suspicious finding in peripheral smear indicated a bone marrow aspiration. Biopsy studies were carried out to exclude silent or latent hematological diseases.

Genetic and other thrombophilic risks were evaluated by determining the levels of factor V Leiden and prothrombin mutations, proteins C and S, antithrombin, IgG and IgM classes of anti-cardiolipin and anti-phospholipid antibodies, homocysteine and fibrinogen. CD 55 and CD 59 clusters in peripheral blood were studied in all patients to rule out paroxysmal nocturnal hemoglobinuria after all hematological investigations.

In view of potential interference with the tests to be used in the study, patients with a history of uncontrolled diabetes and hypertension, severe and widespread atherosclerosis, oral contraceptive use or estrogen replacement treatment, use of anticoagulation drugs, uncontrolled or newly diagnosed malignancy elsewhere, systemic inflammatory diseases and recent (last 3 mo) major operation were excluded. Application of the exclusion criteria reduced the total number of 96 patients (portal vein involvement in 70, hepatic vein involvement in 26) to 9 patients comprised of 7 cases of portal vein thrombosis (PVT) including cavernous transformation of the portal vein, and 2 cases of hepatic vein thrombosis (HVT). After all these investigations, bone marrow biopsies were taken from patients with

Table 2 General characteristics of the patients (results were given as minimum, maximum and median)

| Test | Result |
|------------------------|--------------------------|
| Age | 28-74 (48) |
| Female/Male | 6/3 |
| PVT (n) | 7 |
| HVT (n) | 2 |
| Hemoglobin (gr/L) | 9.5-16.6 (13.5) |
| Platelet (count/mL) | 62.000-358.000 (168.000) |
| ALT (U/L) | 13-38 (23) |
| AST (U/L) | 18-38 (27) |
| ALP (U/L) | 87-355 (200) |
| GGT (U/L) | 16-123 (34) |
| Total Bilirubin (mg/L) | 0.24-2.08 (0.65) |
| INR | 1.06-1.47 (1.14) |
| Protein C (%) | 55-137 (87) |
| Protein S (%) | 58-113 (85) |
| AT (%) | 75-109 (90) |
| Homocysteine (umol/L) | 9-19 (15) |
| Fibrinogen (mg/L) | 181-420 (271) |

a diagnosis of idiopathic thrombosis to rule out any silent hematological diseases.

In excluded patients, the most common thrombophilic conditions were deficiency in proteins C and S and anti-thrombin III (28 patients, 29.1%), followed by myeloproliferative disorders (10 patients, 10.4%), factor V Leiden mutations (10 patients, 10.4%), prothrombin mutations (6 patients, 6.25%). The other conditions related with possible thrombophilia included cyst hydatid disease of the liver, Behcet's disease, metastasis of cancer, mastocytosis invasion of the liver, tuberculosis of the choleduct and abdominal surgery.

The control group comprised of 14 healthy adults (6 males, 8 females) with no history of cigarette smoking, hypertension, atherosclerotic vascular disease, systemic inflammatory condition and no use of medication (oral contraceptives and estrogen replacement treatment). An informed consent was obtained from each patient and control subject. The study protocol received approval of Ethical Committee of Hacettepe University.

Blood samples

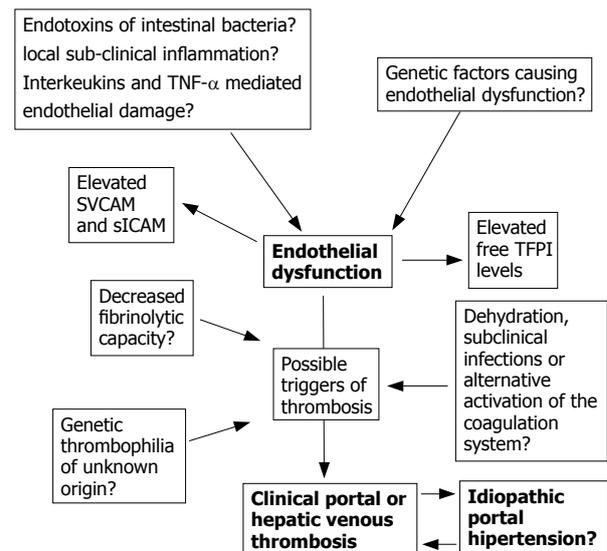
Blood samples from patient and control groups were drawn from antecubital veins with a 20 G needle at 08:00 am after an overnight fasting. All samples were collected in 0.129 mol/L trisodium citrate tubes and centrifuged at 3000 r/min for 10 min, while plasma samples were stored at -30 °C for studies.

Evaluation of endothelial function

The endothelial function was tested by measuring serum concentrations of adhesion molecules, namely sVCAM-1, sICAM-1 and soluble E-selectin. The concentrations of these adhesion molecules were evaluated quantitatively by sandwich enzyme immunoassay (Parameter[®], R&D systems, UK).

Evaluation of initial step of thrombogenesis and its control

The most potent initial step during formation of thrombin, tissue factor-(released from damaged endothelium) related

**Figure 2** Results from our study and possible associations with other processes.

activation of factor VII was evaluated by quantitative measurement of activated factor VII (Staclo[®] VIIa-TF, Diagnostica Stago, Asnieres, France). The major controlling step of this pathway, namely the TFPI (both total and free fractions) level, was tested quantitatively by one-step ELISA (Diagnostica Stago, Asnieres, France).

Fibrinolytic potential

Fibrinolytic capacity of each sample was evaluated by GFC, a semi-quantitative method designed to detect low potentials of given samples. This technique uses the measurement of generated D-dimer in the given sample when a standard amount of fibrin is added into it. The generated D-dimer was measured using macrolatex agglutination (Diagnostica Stago, Asnieres, France). Therefore, the results from this test were semi-quantitatively expressed as "normal GFC", "moderately low GFC" and "severely low GFC".

Statistical analysis

All the data were presented as minimum, maximum and median unless otherwise specified. Statistical tests used for evaluation of significant differences were Mann-Whitney U test and Fisher's exact test.

RESULTS

The general characteristics of the patients are shown in Table 2 and Figure 2. Four patients had portal vein cavernous transformation and 3 patients had pure portal vein thrombosis in the portal vein group, whereas HVT group had only large hepatic vein involvement. As the blood cell count was taken into account, patient group showed a general tendency towards thrombocytopenia due to presence of increased portal venous pressure leading to mild degree of hypersplenism. Liver enzymes were all normal or very slightly elevated owing to the strict exclusion criteria. One important fact was that the international normalized ratio (INR) of the patient group was normal, thus not affecting the hematological tests

Table 3 Comparison of patient and control groups by advanced tests (results were given as minimum, maximum and median)

| Test (normal limits) | Patient group (n) | Control group (n) | Significance |
|------------------------------|-------------------|-------------------|---------------------|
| FVIIa (25.5-96.9 mU/mL) | 27-1232 (96) | 39-325 (129) | NS ¹ |
| Total TFPI (81.2±30.4 ng/mL) | 22-114 (87.2) | 43-112 (79) | NS |
| Free TFPI (10.0±4.8 ng/mL) | 5-41 (19) | 9-20 (13) | ^a P<0.05 |
| sVCAM (395-714 ng/mL) | 489-1890 (915) | 410-890 (517) | ^c P<0.05 |
| sICAM (115-306 ng/mL) | 148-400 (252) | 24-260 (164) | ^b P<0.05 |
| E-Selectin (29.1-63.4 ng/mL) | 14-68 (20) | 11-64 (29) | NS |

¹NS: Not significant; ^aP<0.05, ^cP<0.05, ^bP<0.05 vs control group.

Table 4 Results and comparisons of GFC (global fibrinolytic capacity)

| Group | Severely low GFC | Moderately low GFC | Normal GFC |
|---------------------|------------------|--------------------|------------|
| Patient group | 0 | 4 | 5 |
| Control group | 0 | 0 | 14 |
| Fisher's exact test | | | P<0.05 |

during the study. Proteins C and S showed that the values near the lower normal limits and even mildly low values were due to the decreased synthesis from liver as a result of abnormal blood flow dynamics of liver rather than a congenital deficiency.

The results of hematological evaluation of patient and control groups with advanced tests are shown in Table 3. The comparisons of FVIIa in both groups were similar, so were total TFPI levels. The results obtained from comparisons of free TFPI and adhesion molecules sVCAM-1 and sICAM-1 were striking. Levels of free TFPI, sVCAM-1 and sICAM-1 were significantly higher in patient group than in control group. sE-selectin levels did not differ between the two groups.

The results obtained from patient and control groups by GFC are shown in Table 4. The patient group showed a significant tendency towards moderately low fibrinolysis whereas fibrinolysis was normal in control group.

DISCUSSION

The overall results and our proposals are shown in Figure 2. Firstly, the significant differences in vascular adhesion molecules indicated that there was an overall activation of endothelium comparable to normal subjects, suggesting that there is an unknown abnormality in endothelial functions. Adhesion molecules are synthesized from endothelium in response to bacterial endotoxins, interleukins and inflammatory mediators like tumor necrosis factor- α ^[9-11], indicating that this endothelial dysfunction is a pure result of endothelial dysfunction of splenoportal or hepatic venous systems. Since other external risk factors affecting endothelial function and our tests such as diabetes mellitus, hypertension, cigarette smoking or atherosclerosis were excluded in both patient and control groups, this finding may be of importance.

The comparable difference in free TFPI could be explained also by this endothelial dysfunction. TFPI is a protein molecule formed of three Kunitz particles syn-

thesized and secreted from endothelium. Its main function is to inhibit the activated FVII-tissue factor complex before coagulation cascade becomes uncontrollable^[12,13]. Severe deficiency in TFPI may lead to overt thrombosis in veins^[14]. After synthesized from endothelium, the active component of this protein circulates in free form accounting for approximately 2.5 % of total TFPI pool and it is this free form that exhibits the main TFPI activity whereas the low density lipoprotein-bound TFPI shows no activity at all^[12]. Our findings (a significant difference in free TFPI levels between two groups but no difference in total TFPI levels) were parallel to the findings of endothelial dysfunction confirmed by adhesion molecules, since the increased free TFPI levels indicated a stimulated synthesis from malfunctioning endothelium.

The results obtained from GFC studies indicated that in patient group there was a significant tendency towards moderately low GFC. This in a way is consistent with the recent thrombogenesis theory which accepts that thrombogenesis is not attributable to only one factor. In fact, thrombogenesis requires more than one risk factor for potent initiation of the pathway. From this point, we propose that either low GFC or endothelial dysfunction forms an appropriate milieu for development of this kind of "idiopathic" thrombosis.

The study presented can be questioned in some ways. Firstly, the number of patients studied was small to be conclusive about the hypothesis proposed before. But as discussed before, this patient population was a very rarely confronted group and similar studies are few. Secondly, the patient group had two different vessel involvements and could not be studied as they were homogenous. But if the liver functions and the patterns of thrombus formation were examined in detail, these two different vessel involvements would be similar in terms of idiopathic thrombus formation in milieu of normal liver function. Thirdly, the laboratory tests applied were not informative enough for our assumptions. Formation of thrombus can be evaluated in many ways including quantification of thrombin-antithrombin complexes or prothrombin fragments 1+2 or many other sophisticated tests. However, our hypothesis is not to test the presence of abnormal activation of prothrombin, but to find any possible factors triggering coagulation cascade, resulting in abnormal clot formation. Therefore we tested the most potent initiator of coagulation cascade. It can be argued to study the endothelial functions with other tests like plasminogen activator inhibitor-1 (PAI-1), von-Willebrand factor or thrombomodulin.

To our knowledge, there is no evidence that these tests with adhesion molecules can show endothelial dysfunction. These tests have no superiority over each other for estimation of the degree of endothelial dysfunction.

There is an ongoing debate over the presence of portal vein thrombosis in case of idiopathic portal hypertension (IPH). The pro-thrombogenesis theoreticians believe that there is a tendency towards thrombosis which manifests itself in any life time of a patient with IPH^[15]. The anti-thrombogenesis theoreticians believe that although thrombophilia and repeated micro-thrombi over a long time may be one of the possible etiological factors, the presence of portal thrombosis is a finding against diagnosis of IPH^[16]. We believe that thrombogenesis and silent or unknown risk factors may contribute to the development of IPH^[17].

In conclusion, major idiopathic hepatic thrombosis (hepatic or portal veins) is not as rare as it was thought and our study may be an important clue to further studies in respect of endothelial dysfunction and other possible accompanying coagulation control defects like deficient fibrinolysis.

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RAPID COMMUNICATION

Pulse cyclophosphamide therapy for inflammatory bowel disease

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Abstract

AIM: To assess the efficacy of intravenous cyclophosphamide pulse therapy for refractory inflammatory bowel disease (IBD).

METHODS: We included in our cohort eight patients with (moderate/severe) steroid refractory IBD (4 with ulcerative colitis and 4 with Crohn's disease). They all received 6 cycles of intravenous cyclophosphamide (800 mg) per month.

RESULTS: Patients entered into remission after the second/third cyclophosphamide pulse. Disease activity decreased. There were no side effects and toxicity. All the patients went into long lasting remission. All Crohn's disease patients and 3 of 4 ulcerative colitis patients achieved complete remission. One patient with ulcerative colitis showed an impressive clinical response but did not enter into remission. For the maintenance, patients with Crohn's disease were treated with methotrexate (15 mg/wk) and patients with ulcerative colitis were treated with azathioprine (2.5 mg/kg body weight/d).

CONCLUSION: Remission was maintained in all patients for 6 mo on the average. The drug was well tolerated. These findings suggest that aggressive immunosuppressive therapy may be useful in some refractory patients and further controlled study should be considered in order to fully evaluate this type of treatment as a potential therapy for IBD.

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Key words: Crohn's disease; Ulcerative colitis; Cyclophosphamide

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INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC), collectively referred to as inflammatory bowel disease (IBD), are relatively common inflammatory diseases of the gastrointestinal (GI) tract. Histopathologically and anatomically, these two conditions are distinct, with CD characterized by transmural inflammation that can occur throughout the GI tract, and UC characterized by more superficial inflammation confined to the colon and rectum. The etiology and pathogenesis of IBD are not yet completely understood. Although the details of the pathogenesis of IBD remain elusive, it is believed that exposure of genetically susceptible individuals to environmental factors (e.g., dietary antigens, luminal bacteria) seems to be an important factor for the development and chronicity.

Pharmacotherapy for IBD has essentially been unchanged for over twenty years, with therapy based on 5-aminosalicylic acid (5-ASA) preparations, corticosteroids, antibiotics and immunosuppressants. Many of the controversies about optimal use of these drugs in IBD arise as a consequence of methodological deficiencies in many of the trials combined with the difficulty in consistent patient selection due to the heterogeneous nature of both UC and CD. Current therapy for IBD is neither sufficient nor disease-modifying. The first-line therapy for IBD flares is typically medical in nature. Long-term treatment with non-specific anti-inflammatory drugs such as aminosalicylates, corticosteroids and immunosuppressants is often accompanied with undesirable and potentially serious side effects. Glucocorticoids are a mainstay for the treatment of severe inflammatory bowel disease. Aminosalicylates are efficacious in the treatment of active mild to moderate disease. Infliximab, a chimeric monoclonal anti-TNF alpha antibody can be used in treatment of refractory Crohn's disease^[1]. Novel biologically-driven therapies are targeted to specific pathophysiological processes, offering the potential for better treatment outcomes.

The management of refractory, severe IBD is yet an unsolved problem. Accurate assessment of specific organ involvement and disease severity is vital if we are

going to tailor appropriate therapy for certain patient. In common with other disease, the aims of therapy for IBD fall into three categories, namely induction of remission, maintenance of remission, and prevention of relapse. All should be undertaken with minimal mortality and morbidity either from the disease itself or from the therapy

Cytotoxic drugs prevent cell division or cause cell death. They act predominantly on rapidly dividing cells such as T lymphocytes, and are therefore immunosuppressive and anti-inflammatory. When cytotoxic drugs are initially used in the treatment of cancer, they have profound effects on the immune system. This “unwanted” side effect has subsequently been exploited for the treatment of non-malignant disease where autoimmune mechanisms are considered important in the pathogenesis. Based on the previous observations of improvement in autoimmune diseases (i.e., vasculitides), cyclophosphamide can be the primary cytotoxic drug^[2-4]. Pulse intravenous cyclophosphamide is probably equally effective as oral cyclophosphamide at inducing remission and this remission is usually maintained by continuing cyclophosphamide for 3-6 mo before changing to a combination of other per os therapy. Cyclophosphamide can be administered intravenously at 500-1000 mg/m² pulses monthly. The optimal duration of treatment with intravenous cyclophosphamide has not been determined for IBD, but treatment for six months with monthly pulses would be typical, followed by maintenance therapy with azathioprine or methotrexate. Therapy must be continued to prevent relapse and for maintenance.

Only sporadic but promising cases are reported and experiences with cyclophosphamide in severe IBD are very limited but most patients entering into remission have no major adverse events or side effects^[5]. We here report some other cases to enrich the experiences. We designed a non-controlled prospective pilot study to investigate the effects of pulse cyclophosphamide therapy for IBD patients. The aim of this study was to examine the effect of this kind of therapy on UC and CD.

MATERIALS AND METHODS

Patient selection and treatment

All patients were recruited in this prospective uncontrolled pilot study from our out-patient clinic specializing in chronic inflammatory bowel diseases (at the 3rd Department of Medicine, University of Debrecen). Between September 2002 and December 2003 we included in our cohort eight patients with (moderate/severe) steroid refractory IBD (four patients with CD and four with UC).

All patients were diagnosed according to the standard criteria and underwent endoscopy and/or radiological studies, including ileo-colonoscopy, CT and/or double-contrast barium air enteroclysis during 6 mo of the study. Video endoscopy of the large intestine, including the terminal ileum, was performed before the first cyclophosphamide pulse and at wk 12. All the patients did not respond to conventional therapy (this was the only selection criteria for this study) and were treated with pulse cyclophosphamide therapy monthly (6 cycles). The bolus dose was

applied independent of body weight. Cyclophosphamide (800 mg) was given during a 4 h hospital stay in 500 mL physiologic or normal saline (0.9%) in a period of 120 min. White blood cell (WBC) counts and urine tests for incidental microscopic haematuria were obtained on pre- and post-cyclophosphamide therapy day (0, 7, 14).

Outcome measurement

All the patients were informed of the potential risks and benefits of cyclophosphamide therapy and a written informed consent was given in advance. We defined treatment failure as any of the followings: lack of an initial response, relapse after an initial response, and intolerance to cyclophosphamide necessitating discontinuation of the drug. We used the modified Truelove and Witts activity index for ulcerative colitis and best index (Crohn's disease activity index) for Crohn's disease as others^[6,7]. We used working definitions for the assessment of the response both in UC and CD. Active disease was defined as symptomatic UC with a Truelove-Witts score greater than 6 points and symptomatic CD with a CDAI score greater than 150 points. Significant (moderate-severe) relapse was defined as symptomatic UC with a Truelove-Witts score greater than 10 points and symptomatic CD with a CDAI score greater than 150 points and/or the need for systemic steroid therapy (including oral budesonide), and treatment failure was defined as relapse, colectomy or any serious adverse event.

RESULTS

Patient histories are summarized in Table 1. CD patients were all females aged 36-65 years (mean 49 years) and UC patients were all males aged 29-59 years (mean 40.25 years). Each patient received six monthly treatment. All patients were continued on a daily regimen of methyl prednisolone and the daily dose was decreased according to individual clinical activity. Azathioprine (2.5 mg/kg body weight per day) and methotrexate (15 mg/wk) were initiated orally for UC and CD patients respectively to maintain remission following cyclophosphamide treatment.

The patients entered into remission after the second/third cyclophosphamide pulse. Disease activity decreased. There were no side effects and no toxicity. All the patients went into long lasting remission. Remission seemed stable (except for case 5: relapse after 6 mo). These findings suggested that aggressive immunosuppressive therapy might be useful in some refractory patients and further controlled study should be considered in order to fully evaluate this type of treatment as a potential therapy for IBD.

DISCUSSION

Current therapy for IBD is unsolved. Many drugs are prescribed for inflammatory bowel disease, either for treating active disease or for maintaining remission. However, no drug provides a completely satisfactory response in a large percentage of patients. Corticosteroids (steroids) at dosages equivalent to 40-60 mg/d prednisone are commonly prescribed for acute exacerbations of Crohn's disease and ulcerative colitis. Steroids are not

Table 1 Patient histories

| Patient No | Age/sex | Disease | Duration of IBD (yr) | Localization | CDAI before/after 6 cycles | UC-DAI before/after 6 cycles | Maintenance therapy for remission |
|------------|-----------|---------|----------------------|--------------------|----------------------------|------------------------------|-----------------------------------|
| 1 | 46/female | CD | 26 | colon-rectum | 168/79 | | methotrexate |
| 2 | 65/female | CD | 18 | ileum-colon-rectum | 231/86 | | methotrexate |
| 3 | 49/female | CD | 22 | ileum-colon | 198/91 | | methotrexate |
| 4 | 36/female | CD | 5 | ileum-colon | 242/98 | | methotrexate |
| 5 | 59/male | UC | 5 | rectum | | 14/6 | azathioprine |
| 6 | 44/male | UC | 5 | rectum | | 15/8 | azathioprine |
| 7 | 29/male | UC | 4 | recto-sigmoid | | 13/6 | azathioprine |
| 8 | 29/male | UC | 8 | recto-sigmoid | | 14/7 | azathioprine |

UC-DAI: Modified Truelove and Witts activity index for UC; CDAI: CD activity index; Methotrexate: 15 mg/wk; Azathioprine: 2.5 mg/kg body weight per day.

effective for maintenance of remission in either disease. However, some patients require long-term steroids for suppression of disease, particularly Crohn's disease. Taking steroids for a prolonged period can cause reversible or irreversible adverse effects^[8-10]. These adverse effects are of sufficient concern to prompt consideration of alternative treatment strategies. Infliximab, human growth hormone, and other novel biotechnology treatments have been investigated as therapy for patients with inflammatory bowel disease. Although these biotechnology-derived treatments are promising, their cost is prohibitive for many patients. Because cyclophosphamide is effective for other inflammatory conditions and is relatively less expensive than some other agents, it is considered in the treatment of inflammatory bowel disease.

IBD continues to pose a challenge to clinicians. Over the past few years there have been significant advances in our understanding of its pathogenesis and treatment. These advances may lead to more specific and targeted treatments, with consequent improvements in clinical outcomes. Intravenous pulse cyclophosphamide therapy may be a safe and effective treatment for patients with severe IBD unresponsive to conventional treatment as a first-line adjunct to or replacement of systemic corticosteroids in the treatment of IBD. Last but not the least, costs of this kind of treatment are relatively low.

In conclusion, pulse cyclophosphamide therapy can be used in treatment of selected IBD patients. However, larger placebo-controlled studies in more diverse patient population are warranted.

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Detection of PERV by polymerase chain reaction and its safety in bioartificial liver support system

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sensitive, identified by second PCR. PERVs could be released from hepatocytes cultured in bioreactor without the stimulation of mitogen and could not be prevented by the hollow fiber semipermeable membrane, indicating the existence of PERV safety in extracorporeal bioartificial liver support system (EBLSS).

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Key words: PERV; Bioartificial liver support systems; Polymerase chain reaction

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Abstract

AIM: To establish a method detecting porcine endogenous retrovirus (PERV) in China experimental minipigs and to evaluate the safety of PERV in three individuals treated with bioartificial liver support systems based on porcine hepatocytes.

METHODS: Porcine hepatocytes were isolated with two-stage perfusion method, then cultured in the bioreactor, which is separated by a semipermeable membrane (0.2 μm) from the lumen through which the patients' blood plasma was circulated. After post-hemoperfusion, patients' blood was obtained for screening. Additionally, samples of medium collected from both intraluminal and extraluminal compartments of the laboratory bioreactor and culture supernate *in vitro* was analyzed. The presence of viral sequences was estimated by polymerase chain reaction (PCR) and reverse transcriptase-polymerase chain reaction (RT-PCR). Finally, the infection of virus in the supernate of common culture was ascertained by exposure to the fetal liver cells.

RESULTS: PERV-specific gag sequences were found in the porcine hepatocytes using RT-PCR, and were detected in all samples from the intraluminal, extraluminal samples and culture supernate. However, culture supernatant from primary porcine hepatocytes (cleared of cellular debris) failed to infect human fetal liver cells. Finally, RT-PCR detected no PERV infection was found in the blood samples obtained from three patients at various times post-hemoperfusion.

CONCLUSION: The assays used are specific and

INTRODUCTION

Fulminant hepatic failure (FHF), which remains a disease with high mortality, cannot be reversed by conventional treatments^[1-3]. Meanwhile, the complications of liver failure, such as, encephalopathy, cerebral edema and multi-organ failure made it more difficult to cure. Orthotopic liver transplantation has improved the survival rate to 70%~80% of patients with this almost deadly disease. However, many patients died while waiting for the organ because of the lack of donor liver. Therefore, more curiosity has been aroused to deal this with biological treatments, such as extracorporeal bioartificial liver support system (EBLSS)^[2-6]. The shortage of human organs leads to the use of non-human donors as the main hepatic cell source. Porcine hepatocytes, including transgenic pig, have become an important cell source widely used in bioartificial liver support systems. Concerns have been raised about the safety of this potential therapy, especially the possibility of cross species transmission of porcine endogenous retroviruses (PERV) since *in vitro* infection of human cell lines has been demonstrated in 1997^[4,8-11]. PERVs are integrated into the genome of all pigs. They belong to the gammaretroviruses previously termed type C retroviruses. At least two subtypes of PERV, PERV-A and PERV-B, infect human cells *in vitro*. Viral particles have been shown to be released by mitogen stimulated porcine peripheral blood mononuclear cells (PBMC), *etc*^[10,12-15]. Clinical trials did not find provirus integration, indicating

that no virus infection had taken place. However, further study and more retrospective research should be done in this field.

PERV infection can be measured directly by PCR, RT-PCR^[10,13]. Here, we present the methods for the detection of PERV and investigation of patients who had been treated with EBLSS based on porcine cells. All methods were specific and sensitive, and it was revealed that in all patients no PERV infection had occurred.

MATERIALS AND METHODS

Isolation of porcine hepatocytes

Liver cells were isolated by an adaptation of the two-step perfusion method^[14,19-20]. Briefly, the animals were anesthetized with barbitol (30 mg/kg, b.w, intraperitoneally) and their livers were removed intact. The liver was first perfused *in vitro* via the portal vein with warmed (37 °C) Ca²⁺ and Mg²⁺ free Hanks balanced salt solution at a flow rate of 5-8 mL/min for 10-15 mL/min, and then perfused with 0.05% collagenase (Sigma, Type IV) in the same solution supplemented with 5 mmol/L CaCl₂ and 50 mmol/L HEPES. The reperfusion with collagenase solution lasted 20 min at a rate of 5 mL/min at 37 °C. After 10 min of incubation (37 °C) with gentle shaking, the suspension was filtered and hepatocytes were sedimented at 50×g for 3 min. The viability of the isolated liver cells was determined by the trypan blue exclusion test.

PERV proviral PCR assays

DNA was extracted from porcine liver tissue using the preparation kits (Qiagen). For the detection of provirus, primers specific for the gag gene (forward primer 5'-GCGACCCACGCAGTTGCATA-3', and reverse primer 5'-CAGTTCCTTGCCCAGTGTCCCTT-3') were used, and a second PCR was carried out to test the products using the primers 5'-TGATCTAGTGAGAGAGGCCAGAC-3' and 5'-CGCACACTGGTCCTTGTCG-3'. For PCR amplification, the standard PCR program of one cycle of 95 °C for 10 min, 35 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min and one cycle of 72 °C for 7 min was applied. Liver tissues of human and experimental animals (rabbit and mice) were also gathered to analyze the specificity of PCR.

RT-PCR detection of PERV gag RNA sequences

For the detection of virus released from the cultured cells, viral RNA from supernatants of cultured cells was isolated. Cells were removed from supernate by centrifugation at 200×g for 10 min, thereafter cell debris was removed by centrifugation at 3500×g for 10 min and an additional centrifugation step at 10000×g for 30 min. Viral RNA was extracted using the RNA isolation kits (Qiagen)^[15-18]. RNA was reverse transcribed using a one-step RT-PCR kit (promega) with no-RT PCR controlled.

Porcine hepatocytes cultured in fiber woven bioreactor

Primary cells were harvested from 1 wk China Experimental minipigs by a modified two-step collagenase perfusion method^[19]. After testing the viability of the

cells by trypan blue exclusion, single porcine hepatocyte suspensions were cultivated in the extralumen of the fiber woven bioreactor (developed by our institute (porous, 0.2 μm)). The bioreactor was filled with porcine hepatocyte about 1.0×10⁸~1.4×10⁹ in co-culture with non-parenchymal cells. DMEM containing EGF was circulated with an artificial capillary cell culture system from Cellmax. Both the biological function and enzyme release of the bioreactor were examined.

Patients

Patients included in the study were FHF, which was defined as occurring within 8 wk of onset of the precipitation illness (in the absence of pre-existing liver disease), and acute-on-chronic hepatic failure listed for liver transplantation with a progressive hepatic encephalopathy.

Extracorporeal liver support with the CellMax bioreactor

FHF patients were treated with hybrid liver support system, including a blood circuit with a continuous plasma separation unit and a second circuit for plasma perfusion of the CellMax bioreactor. Briefly, the bioreactor was filled with porcine hepatocyte about 6.0×10⁸-1.4×10⁹ in co-culture with non-parenchymal cells. Venous blood of FHF patients was elicited via a double-lumen dialysis catheter from the internal jugular vein and blood plasma were separated. Continuous hollow fibre plasma separation was performed at a rate of 100-150 mL/min and then stored in sterile chamber. For anticoagulation a continuous infusion of heparin was performed. After one or two days' circulatory culture, the bioreactor was then connected and plasma was continuously perfused at a rate of 150-200 mL/min from the chamber. Treated plasma was reunited with the blood cell and returned to the patients. A roller pump (Millipore ultrafiltration device) was used to circulate blood and a heater was used to maintain the temperature of patients and bioreactor at 37 °C-39 °C. After the perfusion, the patients' plasma samples were detected. Blood samples were obtained after the treatment.

In vitro infection experiments

For infection experiments, cell-free supernatants from cultured porcine hepatocytes were used to infect fetal liver cells in order to mimic the bioartificial liver support system *in vitro*. That is, cell-free supernatants from primary cultured porcine hepatocytes were collected and cell debris was removed by centrifugation at 10000× for 30 min, then was used to infect fetal liver cells.

RESULTS

Cell yield and viability

The total isolated hepatocytes and non-parenchymal liver cells by the simplified two step perfusion method were 6.0-14.0×10⁸ cell per liver. The estimated viability judged by the trypan blue test was 92%-96%.

Specificity of the PERV DNA assays

All these PCR assays gave negative results on tissue and serum samples from 2 HBV patients, as well as 2 HCV

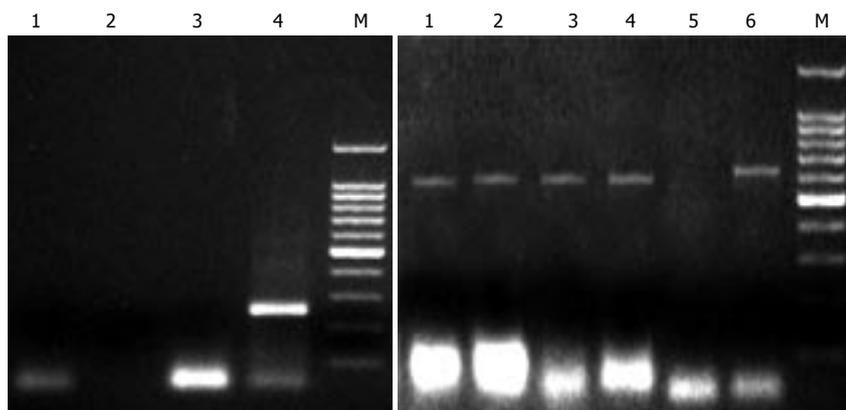


Figure 1 PCR analysis of PERV proviral DNA in different liver tissues. Left: 1: normal person liver tissue, 2: HBV positive liver tissue, 3: HCV positive liver tissue, 4: second PCR of pig liver tissue, 5: Marker. Right: 1, 2, 3, 4: pig liver cells, 5: negative control.

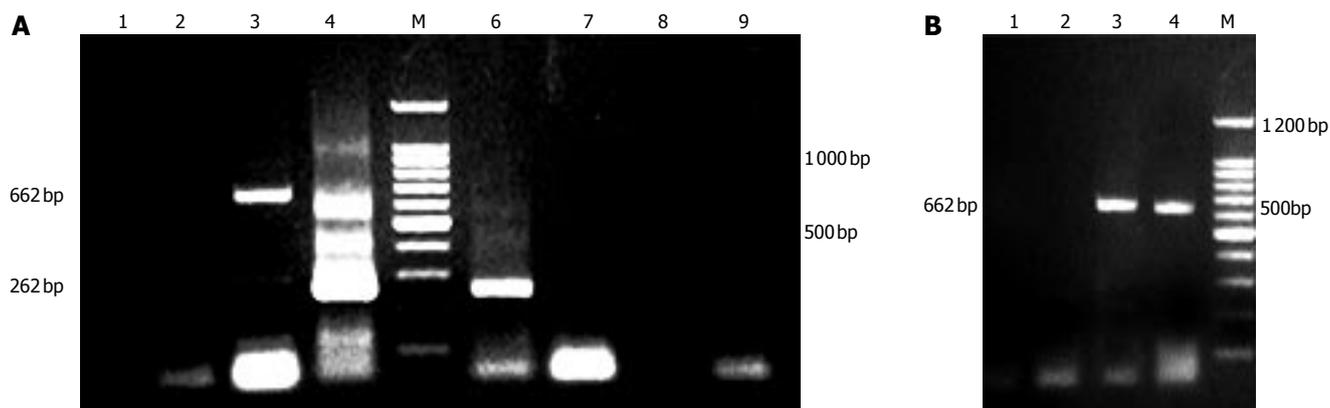


Figure 2 A: PERV RNA detection of supernate. 1:supernate of d 1, 2: supernate of d 3; 3:no RT PCR of d 3; 4:second PCR of d 3; 6: second PCR of d 5; 7: RT-PCR of d 7; 8: RT-PCR of d 9; 9: no RT PCR of d 9; **B:** PERV permeation of bioreactor. 1: intraluminal supernate of d 3 (no RT PCR), 2: extraluminal supernate of d 3 (no RT PCR), 3: intraluminal supernate of d 3 (RT-PCR); 4: extraluminal supernate of d 3 (RT-PCR).

patients and 1 rabbit, and positive results on tissue of China experimental swine, demonstrating 100% sensitivity, as shown in Figure 1. The detection of product with second PCR gave the same results, showing the usefulness and specificity of the method in observing PERV infection.

PERV release of cultured porcine hepatocytes in bioreactor

As depicted in Figure 2A, PERV could be released from porcine hepatocytes cultured not only in common flasks but also in fiber woven bioreactor without the stimulation of mitogen. In the early stage, the quantity of virus so small that it couldnot be detected by PCR assays. From third day on, a large amount of PERVs could be released from the cells till their death. We also found that the semipermeable membrane (0.2 um) of bioreactor couldnot separate the virus, because in the both sides of the lumen we had evidence of the existence of PERV (Figure 2B).

PERV detection of patients using EBLSS

Continuous EBLSS treatment over a period of 4-6 h was safely performed and well tolerated by all the patients. No complications associated with therapy were observed during the treatment and the follow-up period of 1-2 years. After treatment, patients' blood was obtained for screening with the method mentioned above. No PERV RNA was detected in the serum of any of the 3 patients (Figure 3).

PERV infection experiments in vitro

PERV infection experiments with human fetal liver cells were performed. Supernatants from the cultured porcine hepatocytes were positive for PERV detection. Although it has been demonstrated that human kidney cells can be infected, it was also important to evaluate PERV infection of other human cells. In all fetal cells at one week post-infection, no evidence showed that PERV infection occurred with PCR and RT-PCR assays (Figure 4).

DISCUSSION

EBLSS based on porcine hepatocytes catches more interest as an effective temporary treatment to improve the chance of survival and a bridge to liver transplantation^[5,6,20-24,38-41]. Because of the shortage of human cells, porcine endogenous retroviruse (PERV), found in 1970's, was focused for its safety in xenotransplantation and treatments based on porcine tissues or cells as vitro infection of human cell lines HEK 293 was demonstrated in 1997^[4,34-37]. Laboratory surveillance of PERV infection in pig xenograft recipients and the treatments is critical for determining the safety of pig xenotransplantation. We used here highly specific PCR based assays for the molecular detection of PERV DNA and RNA sequences. In addition, we included a control PCR reaction without RT to confirm that the positive RT-

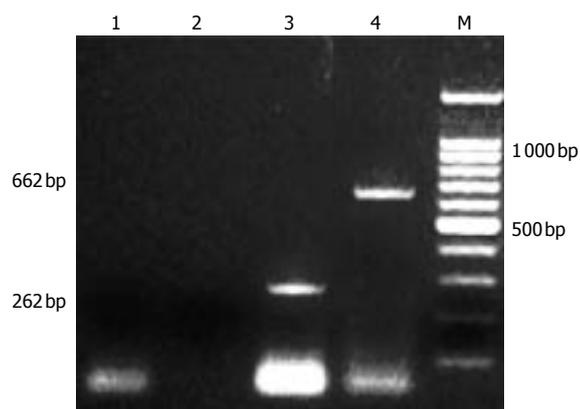


Figure 3 infection of PERV. 1,2 RT-PCR and second PCR of cultured fetal hepatocytes, 3,4: positive control.

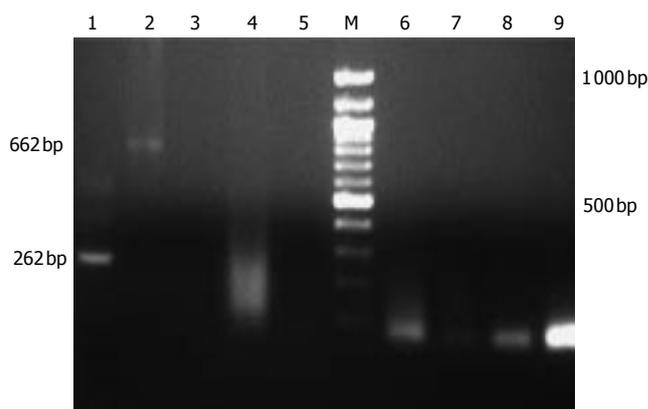


Figure 4 PERV detection of patients treated with EBLSS. 1:second PCR of positive control, 2: positive control, 3:control; 4-9:RT-PCR and second PCR of patients.

PCR results were due to PERV RNA. we have developed polymerase chain reaction (PCR) and reverse transcription polymerase chain reaction (RT-PCR) assays to detect proviral PERV gag sequences and PERV RNA sequences in serum samples by using specific primers. All these PCR assays gave positive results on tissue of China experimental swine, and the identification of product with second PCR gave the same results, showing the sensitivity and specificity of this method in observing PERV infection.

Viral particles have been shown to be released by pig PBMC, by cultured aorta endothelial cells stimulated with mitogen^[10,12,13,25,26]. In several trials, human diabetic patients who were treated with pig pancreatic islet cells, with pig skin or with extracorporeal perfusion of pig liver did not show provirus integration^[27,42], indicating that no virus infection had taken place. *In vitro* infection experiments revealed that PERVs infect not only human cell lines and primary cells, but also a variety of cells of other species including non-human primates. However, no transmission of PERVs *in vivo* was observed in non-human primates and several small animals gave high doses of PERV. Additional data showing the same results about the safety of PERV have been obtained from several experiments^[16,29]. Nevertheless, considering the results of initial clinical xenotransplantations in humans, which found that persistent microchimerism was observed in 23 patients (totally 160) for up to 8.5 years, and the establishment of PERV infection model^[27,28], it was worth paying more attention to its safety in xenotransplantation and EBLSS.

In this study, the retrospective investigation of the three patients with the treatment of EBLSS based on porcine hepatocytes was performed for possible PERV transmission. Our data are in agreement with other studies showing lack of PERV transmission. And in laboratory experiment we also found PERV could not be prevented by the hollow fibre membranes, indicating different opinion from the foregoing research that PERV particles could be prevented by using PES hollow fibre membranes with a molecular weight cut-off of 400 000 kD^[31]. In fact, the pore size of bioreactor should be large enough to make material exchange possible, so, any efforts trying to block

the virus with semipermeable membrane was probably in vain. The lack of PERV transmission in the patients investigated could be owing to short-term contacting with the EBLSS, lasting for 4-6 h during the treatments. And an effective inactivation of PERV released from pig cells may be another reason. Although cells of human could be infected productively with PERV as reported, no transmission was observed in patients treated with EBLSS in our study.

The latest data about the safety of xenotransplantation using porcine cells, tissues and organs has been obtained from experiments involving transplantation of encapsulated pig islet cells into diabetic rats and assessment of PERV infection in patients treated with a bioreactor based on porcine liver cells^[29-33], demonstrating no PERV transmission occurred. Although no data indicated that PERV infection had occurred in any of the patients treated with the BALSS containing porcine hepatocytes, longer and larger patient follow-up is required to supervise porcine retroviruses and EBLSS.

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Clinical correlation of gallstone disease in a Chinese population in Taiwan: Experience at Cheng Hsin General Hospital

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CI: 2.43-9.99], 60-69 years vs <40 years, OR = 6.82 [95% CI: 3.19-14.60], ≥ 70 years vs <40 years, OR = 10.65 [95% CI: 4.78-23.73]), higher BMI (≥ 27 kg/m² vs <24 kg/m², adjusted OR = 1.74 [95% CI: 1.04-2.88]), and higher FPG (≥ 126 mg/dL vs <110 mg/dL, OR = 1.71, 95%CI: 1.01-2.96).

CONCLUSION: Older age (≥ 50 years), obesity (BMI ≥ 27 kg/m²), and type 2 diabetes (FPG ≥ 126 mg/dL) are associated with the prevalence of GSD.

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Key words: Cross-sectional study; Gallstone disease; Prevalence; Risk factors

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Abstract

AIM: To explore the prevalence of gallstone disease (GSD) in Taiwan and condition-associated factors related to it.

METHODS: We studied a total of 2386 healthy adults (1235 males and 1151 females) voluntarily admitted to Cheng Hsin General Hospital for a paid physical check-up between January 2002 and December 2002. Blood samples and ultrasound sonography results were collected.

RESULTS: The overall prevalence of GSD among this study-population was 5.3%, including 1.7% ($n = 40$) having a single stone, 2.3% ($n = 55$) having multiple stones, and 1.3% ($n = 31$) having cholecystectomy. The prevalence revealed a statistically significant increase with increasing age ($P < 0.0001$). Females exhibited a greater prevalence of multiple stones than did males (3.0% vs 1.7%, $P = 0.04$). Using multiple logistic regression analysis, the following appeared to be significantly related to the prevalence of GSD: older age (40-49 years vs <40 years, OR = 1.63 [95% CI: 0.76-3.48], 50-59 years vs <40 years, OR = 4.93 [95%

INTRODUCTION

Gallstone disease (GSD) is one of the most common diseases in developed countries. Recent studies indicate varying prevalence of GSD with several predisposing factors in different study populations^[1-9]. From a medical economic perspective, the direct and indirect costs of treating GSD patients were estimated at \$16 billion and account for more than 800 000 hospitalizations yearly in the United States^[9,10]. In Taiwan, the prevalence of GSD in the general population was 4.3% in 1989^[8] while another voluntary screening revealed that the prevalence of GSD was 10.7% among healthy subjects in 1995^[2]. For this reason, due to westernization of the diet and the environment, GSD is not rare in the Chinese population and has become one of the major health problems in Taiwan^[7]. Without an appropriate and effective screening program for symptomatic GSD, the medical treatment of GSD and related complications contributes substantially to health care costs.

From the viewpoint of preventive medicine, it is

Table 1 Prevalence of each type of gallstone disease among 2386 Chinese subjects

| Variable | Gallstone disease | | | |
|---|-----------------------|------------------------------------|---------------------------------------|---------------------------------------|
| | Total screened number | Single stone prevalence number (%) | Multiple stones prevalence number (%) | Cholecystectomy prevalence number (%) |
| Gender | | | | |
| Male | 1235 | 21 (1.7) | 21 (1.7) | 17 (1.4) |
| Female | 1151 | 19 (1.7) | 34 (3.0) | 14 (1.2) |
| <i>P</i> -value for χ^2 -test | | 0.95 | 0.04 | 0.76 |
| Age | | | | |
| <40 | 745 | 4 (0.5) | 5 (0.7) | 2 (0.3) |
| 40-49 | 723 | 6 (0.8) | 7 (1.0) | 6 (0.8) |
| 50-59 | 504 | 11 (2.2) | 21 (4.2) | 8 (1.6) |
| 60-69 | 252 | 7 (2.8) | 17 (6.8) | 6 (2.4) |
| 70+ | 162 | 12 (7.4) | 5 (3.1) | 9 (5.6) |
| <i>P</i> -value for Cochran-Armitage trend test | | <0.0001 | <0.0001 | <0.0001 |
| Total | 2386 | 40 (1.7) | 55 (2.3) | 31 (1.3) |

not only important to be cognizant of the background prevalence of GSD regionally, but also to explore the complete spectrum of demographic and biological markers which may be related to development of GSD. Although most epidemiologic studies of GSD, demographic factors and biochemical markers have used case-control designs and cross-sectional data^[1-9], to the best of our knowledge, some uncertainty still exists regarding the prevalence of the disease and the identity of the associated risk factors for the development of GSD. The present study was designed to explore potential associated risk factors and to improve understanding of the overall pathogenesis of GSD. The purpose of this study was to explore the context of associated risk factors for GSD prevalence amongst the general population aged 20 years or more, as determined by the application of a healthy volunteer subjects screening program at Cheng Hsin General Hospital, a fully certified regional hospital and teaching hospital in Taipei, Taiwan.

MATERIALS AND METHODS

Data resource and data collection

This cross-sectional study was conducted with a total of 2386 healthy adults (1235 males and 1151 females) voluntarily admitted to Cheng Hsin General Hospital for a paid physical check-up between January 2002 and December 2002. Blood samples and ultrasound sonography results were collected. Overnight-fasting blood samples were drawn via venipuncture from study participants by clinical nurses. Serum and plasma samples (from whole blood preserved with EDTA and NaF) were kept frozen (-20 °C) until ready for analysis. The study-used definition of type-2 diabetes was from the 1997 ADA criteria^[11]. Definitions of the following diseases / conditions were obesity: a body mass index (BMI) ≥ 27 Kg/m², high systolic blood pressure (SBP) ≥ 140 mmHg, high diastolic blood pressure (DBP) ≥ 90 mmHg, hyper-

cholesterolemia (≥ 200 mg/dL), hypertriglyceridemia (≥ 200 mg/dL), low HDL (< 35 mg/dL), high BUN (≥ 20 mg/dL), high creatinine (≥ 1.4 mg/dL), and hyperuricemia (≥ 7 mg/dL for males or ≥ 6 mg/dL for females). Serum ALT or AST levels ≥ 40 U/L were classified as elevated^[12]. In addition, information on HBV and anti-HCV were collected using radioimmunoassay kits.

Diagnosis of gallstone disease

In the present study, GSD was diagnosed by a panel of specialists using real-time ultrasound sonography (TOSHIBA nemio SSA-550A, Japan) to examine the abdominal region after fasting for at least 8 h based on the presence of "movable hyper-echoic foci with acoustic shadow. Cases of GSD were classified as follows: single gallbladder stone, multiple gallbladder stones, and cholecystectomy, excluding gallbladder polyps. Cases were identified as any type of GSD study population.

Interobserver reliability in ultrasound sonography

In order to set up a consistent diagnosis of GSD between specialists, the Kappa statistic was used to assess the agreement of inter-observer reliability among study specialists. A pilot study was performed using 100 randomly selected healthy subjects other than the study participants. For inter-observer reliability, the Kappa value for diagnosis of GSD between specialists was 0.79 (95%CI: 0.61-0.95).

Statistical analysis

Statistical analysis was performed using SAS for Windows, (SAS version 9.0; SAS Institute Inc., Cary, NC, USA). A *P*-value of < 0.05 was considered statistically significant between two test populations. For univariate analysis, the two-sample, independent t-test method was adopted to assess differences in the mean value of continuous variables between subjects with and without GSD. Crude and adjusted odds ratios (adjustment for gender and age) were estimated and 95% confidence intervals were used. Multiple logistic regression was also performed in order to investigate the independence of risk factors associated with the prevalence of GSD.

RESULTS

The prevalence of each type of GSD amongst study subjects is presented in Table 1. The overall prevalence of GSD was 5.4%, including 1.7% ($n = 40$) having a single stone, 2.3% ($n = 55$) having multiple stones, and 1.3% ($n = 31$) having cholecystectomy. Females (3.0%) had a significantly higher prevalence of multiple stones than males (1.7%) ($P = 0.04$). There were no statistically significant differences in gender for other types of GSD. In addition, from the Cochran-Armitage trend test, the prevalence of each type of GSD showed an increase with age ($P < 0.0001$). Subjects aged 50 years and over ($96/918 = 10.5\%$) had a more than 5-fold risk for GSD compared with the subjects aged 50 years and less ($30/1468 = 2.0\%$).

Figure 1 shows the gender- and age-specific prevalence of all types of GSD. Although there was no gender difference ($\chi^2 = 1.30$, $P = 0.25$) for the overall prevalence

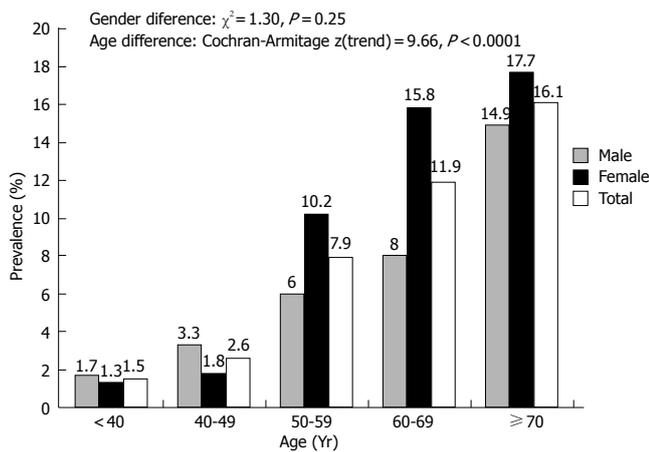


Figure 1 Gender- and age-specific prevalence of all types of gallstone disease among 2386 Chinese subjects.

Table 3 Univariate analysis of associated clinical factors for gallstone disease among 2386 Chinese subjects

| Variable | Gallstone disease | | Crude OR (95% CI) | Adjusted OR ¹ (95% CI) |
|------------------------------|-------------------|---------------|--------------------|-----------------------------------|
| | Yes (n = 126) | No (n = 2260) | | |
| Gender male | 59 | 1176 | 1.00 | - |
| female | 67 | 1084 | 1.23 (0.86-1.76) | - |
| Age (yr) <40 | 11 | 734 | 1.00 | - |
| 40-49 | 19 | 704 | 1.80 (0.85-3.81) | - |
| 50-59 | 40 | 464 | 5.75 (2.92-11.32) | - |
| 60-69 | 30 | 222 | 9.02 (4.45-18.29) | - |
| ≥70 | 26 | 136 | 12.76 (6.16-26.43) | - |
| BMI (Kg/m ²) <24 | 57 | 1343 | 1.00 | 1.00 |
| 24-27 | 38 | 585 | 1.53 (1.00-2.33) | 1.36 (0.88-2.10) |
| ≥27 | 31 | 332 | 2.20 (1.40-3.46) | 2.04 (1.28-3.26) |
| FPG (mg/dL) <110 | 93 | 2007 | 1.00 | 1.00 |
| 110-125 | 10 | 122 | 1.77 (0.90-3.49) | 1.14 (0.57-2.28) |
| ≥126 | 23 | 126 | 3.95 (2.42-6.45) | 1.95 (1.16-3.30) |
| High SBP No | 93 | 1885 | 1.00 | 1.00 |
| Yes | 33 | 375 | 1.78 (1.18-2.69) | 1.18 (0.54-1.33) |
| High DBP No | 97 | 1801 | 1.00 | 1.00 |
| Yes | 29 | 459 | 1.17 (0.77-1.80) | 0.95 (0.61-1.48) |
| Hypercholesterolemia No | 93 | 1827 | 1.00 | 1.00 |
| Yes | 33 | 433 | 1.50 (1.00-2.26) | 1.11 (0.72-1.69) |
| Hypertriglyceridemia No | 100 | 1946 | 1.00 | 1.00 |
| Yes | 26 | 314 | 1.61 (1.03-2.52) | 1.54 (0.97-2.46) |
| Low HDL No | 122 | 2052 | 1.00 | 1.00 |
| Yes | 4 | 208 | 0.32 (0.12-0.89) | 0.62 (0.22-1.73) |
| High BUN No | 115 | 2118 | 1.00 | 1.00 |
| Yes | 11 | 142 | 1.43 (0.75-2.71) | 0.67 (0.34-1.33) |
| High creatinine No | 118 | 2214 | 1.00 | 1.00 |
| Yes | 8 | 46 | 3.26 (1.51-7.07) | 1.22 (0.54-2.78) |
| Hyperuricemia No | 43 | 963 | 1.00 | 1.00 |
| Yes | 83 | 1297 | 1.43 (0.98-2.09) | 1.18 (0.80-1.75) |
| Higher AST No | 106 | 2095 | 1.00 | 1.00 |
| Yes | 20 | 165 | 2.40 (1.45-3.97) | 1.96 (1.16-3.32) |
| Higher ALT No | 101 | 1883 | 1.00 | 1.00 |
| Yes | 25 | 377 | 1.24 (0.79-1.94) | 1.48 (0.92-2.37) |
| HBV Negative | 113 | 1975 | 1.00 | 1.00 |
| Positive | 13 | 285 | 0.80 (0.44-1.43) | 0.98 (0.54-1.78) |
| Anti-HCV Negative | 123 | 2204 | 1.00 | 1.00 |
| Positive | 3 | 56 | 0.96 (0.30-3.11) | 0.63 (0.19-2.07) |

¹Adjustment for gender and age.

Table 2 Comparison of characteristics in subjects with and without gallstone disease

| Variable | Any type of gallstone disease | | | P-value for <i>t</i> test |
|--------------------------|-------------------------------|-----------------|------------------|---------------------------|
| | Yes (n = 126) | No (n = 2260) | Total (n = 2386) | |
| Age (yr) | 58.63 ± 13.04 | 46.57 ± 13.22 | 47.21 ± 13.48 | <0.0001 |
| BMI (kg/m ²) | 24.83 ± 3.64 | 23.50 ± 3.55 | 23.57 ± 3.57 | <0.0001 |
| FPG (mg/dL) | 109.22 ± 33.21 | 96.98 ± 25.21 | 97.63 ± 25.84 | <0.0001 |
| SBP (mmHg) | 130.06 ± 20.07 | 122.55 ± 18.40 | 122.94 ± 18.56 | <0.0001 |
| DBP (mmHg) | 81.27 ± 12.22 | 79.56 ± 12.56 | 79.65 ± 12.55 | 0.14 |
| Cholesterol (mg/dL) | 216.93 ± 41.06 | 210.51 ± 37.72 | 210.87 ± 37.94 | 0.07 |
| Triglyceride (mg/dL) | 143.39 ± 98.63 | 130.12 ± 108.06 | 130.86 ± 107.58 | 0.18 |
| HDL (mg/dL) | 57.02 ± 16.55 | 57.69 ± 15.76 | 57.65 ± 15.80 | 0.64 |
| BUN (mg/dL) | 14.94 ± 6.28 | 13.55 ± 5.56 | 13.63 ± 5.61 | 0.02 |
| Creatinine (mg/dL) | 1.08 ± 0.35 | 1.04 ± 0.40 | 1.04 ± 0.40 | 0.29 |
| Uric acid (mg/dL) | 6.74 ± 1.67 | 6.59 ± 1.72 | 6.60 ± 1.72 | 0.35 |
| AST (U/L) | 32.79 ± 30.13 | 27.59 ± 17.59 | 27.88 ± 18.54 | 0.06 |
| ALT (U/L) | 37.84 ± 54.27 | 30.01 ± 34.49 | 30.45 ± 35.91 | 0.11 |

of GSD, there was a statistically significant increase with increasing study-subject age by means of the trend test ($z = 9.66, P < 0.0001$). The prevalence of GSD also showed an interaction effect between gender and age, that is, males proved to have a substantially greater overall prevalence of GSD than females among those aged less than 50 years, whereas there was a lower overall prevalence of GSD among those aged more than 50 years.

The results of the comparison of a variety of test characteristics and their potential association with the prevalent GSD for study subjects are illustrated in Table 2. Using a two-sample independent *t*-test, the associated factors that were significantly related to GSD included age (yes [58.63 ± 13.04 years] *vs* no [46.57 ± 13.22 years]), BMI (yes [24.83 ± 3.64 Kg/m²] *vs* no [23.50 ± 3.55 Kg/m²]), fasting plasma glucose (FPG) (yes [109.22 ± 33.21 mg/dL] *vs* no [96.98 ± 25.21mg/dL]), SBP (yes [130.06 ± 20.07 mmHg] *vs* no [122.55 ± 18.40 mmHg]), and BUN (yes [14.94 ± 6.28 mg/dL] *vs* no [13.55 ± 5.56 mg/dL]).

Table 3 presents the crude and adjusted odds ratios for the association between certain relevant associated risk factors and the prevalence of GSD. Compared to individuals without GSD, in addition to older age (40 - 49 years *vs* <40 years, OR = 1.80 [95% CI: 0.85 - 3.81], 50-59 years *vs* <40 years, OR = 5.75 [95% CI: 2.92-11.32], 60-69 years *vs* <40 years, OR = 9.02 [95% CI: 4.45-18.29], ≥ 70 years *vs* <40 years, OR=12.76 [95% CI: 6.16-26.43]), subjects featuring GSD revealed a more-pronounced prevalence of: higher BMI (≥27 kg/m² *vs* <24 kg/m², adjusted OR = 2.04 [95% CI: 1.28-3.26]), higher FPG (≥ 126 mg/dL *vs* <110 mg/dL, OR = 1.95, 95% CI: 1.16-3.30), and higher AST (adjusted OR=1.96, 95%CI: 1.16-3.32) subsequent to adjustment for gender and age.

The effect of independent associated risk factors on GSD was examined using a multiple logistic regression model. As depicted in Table 4, subsequent to adjustment for confounding factors, the following appeared to be significantly related to GSD prevalence: age (40-49 years *vs* <40 years, OR = 1.63 [95% CI: 0.76-3.48], 50-59 years *vs*

Table 4 Multiple logistic regression of associated factors for gallstone disease among 2386 Chinese subjects

| Variable | Gallstone disease (yes vs no) | |
|---------------------------------------|----------------------------------|------------|
| | OR | 95%CI |
| Gender (female vs male) | 1.34 | 0.90-1.99 |
| Age (40-49 vs <40 yr) | 1.63 | 0.76-3.48 |
| (50-59 vs <40 yr) | 4.93 | 2.43-9.99 |
| (60-69 vs <40 yr) | 6.82 | 3.19-14.60 |
| (≥70 vs <40 yr) | 10.65 | 4.78-23.73 |
| BMI (24-27 vs <24 kg/m ²) | 1.25 | 0.79-1.97 |
| (≥27 vs <24 kg/m ²) | 1.74 | 1.04-2.88 |
| FPG (110-125 vs <110 mg/dL) | 0.96 | 0.47-1.97 |
| (≥126 vs <110 mg/dL) | 1.71 | 1.01-2.96 |
| High SBP (yes vs no) | 1.11 | 0.53-1.51 |
| High DBP (yes vs no) | 0.87 | 0.52-1.48 |
| Hypercholesterolemia (yes vs no) | 0.99 | 0.64-1.53 |
| Hypertriglyceridemia (yes vs no) | 1.25 | 0.75-2.08 |
| Low HDL (yes vs no) | 0.5 | 0.17-1.44 |
| High BUN (yes vs no) | 0.59 | 0.26-1.37 |
| High creatinine (yes vs no) | 2.02 | 0.71-5.75 |
| Hyperuricemia (yes vs no) | 0.97 | 0.64-1.46 |
| Higher AST (yes vs no) | 2.06 | 0.99-4.31 |
| Higher ALT (yes vs no) | 0.83 | 0.42-1.63 |
| HBV (positive vs negative) | 0.95 | 0.52-1.76 |
| Anti-HCV (positive vs negative) | 0.5 | 0.15-1.69 |

<40 years, OR = 4.93 [95%CI: 2.43 - 9.99], 60 - 69 years vs <40 years, OR = 6.82 [95% CI: 3.19 - 14.60], ≥70 years vs <40 years, OR = 10.65 [95% CI: 4.78 - 23.73]), the higher BMI (≥27 kg/m² vs <24 kg/m², adjusted OR = 1.74 [95% CI: 1.04 - 2.88]), and higher FPG (≥126 mg/dL vs <110 mg/dL, OR = 1.71, 95% CI: 1.01 - 2.96).

DISCUSSION

Prevalence of gallstone disease in the general population

One of the important benefits of early screening for GSD is that ultrasonography can lead to the discovery of an increasing proportion of asymptomatic cases for which one can use therapeutic nonsurgical approaches, such as litholytic bile acids, local solvents, and lithotripsy^[13]. However, it appears that only a few published studies have attempted to determine the prevalence and possible etiology of GSD prevalence for the general population of Taiwan^[2,8,14], which also face a GSD burden. In the present study, GSD appeared to be fairly common for the test population, affecting an estimated 5.3% of the general population in Taiwan. The prevalence of GSD amongst different test populations appears to vary, differing among different studies conducted in different countries^[1,3-6]. In addition to different methods of GSD assessment, this disparity might be due to differences between different population stocks. The prevalence of GSD for our study population (5.3%) was lower than the corresponding figure presented in a previous hospital-based study conducted in the Veterans General Hospital (VGH), Taipei, Taiwan, which was reported to be 10.7%^[2]. The apparent lower prevalence rate in our study may have been due to younger age of the participants. Another possible reason for such

differences between the results of the VGH study and our results may simply have been related to the fact that although the kappa value for agreement of interobserver reliability seemed good^[14], non-differential misclassification-bias identification could still be occurring and could lead to underestimation of GSD prevalence.

The implications of associated risk factors for gallstone disease

Our results reveal that older age represents a significant risk factor for GSD. Such a finding is consistent with results of other hospital-based and community-based studies conducted elsewhere^[2-5,7,8]. GSD is very seldom found in children, and in that age group is mainly associated with haemolytic causes^[15]. The long-term exposure to many other risk factors among elder persons may also account for the increased probability of developing GSD. Cholelithiasis is also an acquired disease contributed to by chronic environmental factors plus an aging effect^[7]. In addition, it has been suggested that among the elderly, larger amounts of cholesterol are secreted by the liver, and the catabolism of cholesterol to bile acid is decreased^[16,17].

Most previous epidemiologic studies have shown that females have a higher prevalence of GSD than males in the Western world. However, the male to female ratio seems to have changed from early reports, which showed figures of 1:4-6 to more recent studies where the ratio is 1:2 or less^[3,4,18]. Pregnancy and sex hormones could be involved by altering biliary secretion or gallbladder motility, or both may play an important role in sex-related differences in the prevalence of GSD^[13]. Another possible reason might be that estrogen replacement therapy or oral contraceptives are used^[19]. Our findings showed that, except for multiple stones, females do not have a significantly higher prevalence of all types of GSD than males. This result is similar to that from other hospital-based or population-based studies conducted in Taiwan^[2,7,8].

After adjustment for confounding factors, the present study also suggests that obesity is highly correlated with GSD prevalence. Supersaturated bile is the linkage between obesity and cholesterol GSD. Obesity can raise the saturation of bile by increasing biliary secretion of cholesterol - the latter probably depending on a higher synthesis of cholesterol in obese subjects^[7,13,20]. While cholesterol GSD is common in Western countries, pigment GSD is still the principal component in Taiwan^[2]. Our results also implied that obesity may be an independent associated risk factor leading to prevalent pigment GSD in the Chinese population.

It has been a matter of controversy whether diabetes mellitus is associated with GSD or not^[7]. Consistent with previous studies^[4,7], our results showed that diabetes mellitus is highly associated with GSD prevalence. From a clinical perspective, hyperglycemia inhibits bile secretion from the liver and disturbs gallbladder contraction^[2]. Supersaturated bile in the gallbladder induces cholelithiasis in diabetic subjects^[21]. The association of diabetics with GSD is stronger in subjects with a history of treated diabetes mellitus than in those with a simple history of diabetes and this could be an effect of hyperglycemia on gallbladder motility^[22]. Furthermore, diabetes mellitus

combined with GSD may induce acute cholecystitis more frequently and have a higher possibility of progression to septicemia^[7].

An increasing prevalence of cholelithiasis has been associated with the etiology and severity of chronic liver disease and cirrhosis^[23]. Possible explanatory factors include overproduction and over-secretion of bilirubin due to increased extracorporeal hemolysis, a reduction in the production and transportation of biliary salts and cholesterol secretion, stasis and changes in bile acidification, or even mucous hypersecretion and subtle alterations in gallbladder mucosal function^[24,25]. However, although early liver dysfunction such as abnormal ALT, HBV, and HCV infection are endemic in Taiwan^[12,26,27], the present study did not reveal liver function associated factors to be related to GSD prevalence. This finding is consistent with other hospital-based studies for the general population in Taiwan^[2]. Further epidemiological and etiologic investigations are needed to clarify the pathophysiological mechanisms between early liver damage and pigment GSD among general Chinese populations.

Perceived limitations

A major limitation of the present study is the potential self-selection bias due to the hospital-based study design, that is, of it not being exactly representative of the whole general population. Second, it is well established that a large proportion of GSD patients remain asymptomatic and they are therefore often ignored for many years. Thus the present study may be representative of the clinical and not of the true prevalence. Thirdly, we do not detail to distinguish between cholesterol stones and pigment stones in this study, some measurement errors and different pathogenicity could occur. Fourthly, we did not consider how many individuals had progressive liver disease and explore the relationship between GSD and liver disease. Finally, our measurements were done only at a single time point and could not be able to reflect long-term exposure to various demographic or biochemical aspects or factors, factors which might be important influencers of GSD. The solution to such a quandary is to conduct a number of prospective longitudinal analogous studies, the results of which would be expected to complement the cross-sectional findings of this study.

CONCLUSION

In conclusion, older age, obesity (BMI ≥ 27 kg/m²), and type 2 diabetes (FPG ≥ 126 mg/dL) are associated with GSD prevalence. Further studies are needed not only to elucidate the temporal sequence of events that typically lead to GSD, but also to further explore the pathogenesis of GSD in the general Chinese population.

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RAPID COMMUNICATION

Correlation between ultrasonographic and pathologic diagnosis of liver fibrosis due to chronic virus hepatitis

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Abstract

AIM: To evaluate the validity of ultrasonographic and pathologic diagnosis of liver fibrosis in patients with chronic viral hepatitis.

METHODS: The liver fibrosis status in 324 patients was evaluated by both needle biopsy and ultrasonography. Liver fibrosis was divided into S0 -S4 stages. S4 stage was designated as definite cirrhosis. The ultrasonographic examination included qualitative variables, description of liver surface and parenchyma, and quantitative parameters, such as diameter of vessels, blood flow velocity and spleen size.

RESULTS: Ultrasonographic qualitative description of liver surface and parenchyma was related with the severity of fibrosis. Among the quantitative ultrasonographic parameters, cut-off value of spleen length (12.1 cm) had a sensitivity of 0.600 and a specificity of 0.753 for diagnosis of liver cirrhosis. The diameters of spleen (8 mm) and portal vein (12 mm) had a diagnostic sensitivity of 0.600 and 0.767, and a diagnostic specificity of 0.781 and 0.446, respectively. The diagnostic accuracy for liver cirrhosis was moderately satisfactory, and the negative predictive values of these parameters reached near 0.95.

CONCLUSION: Ultrasonography can predict the degree of liver fibrosis or cirrhosis. A single ultrasonographic parameter is limited in sensitivity and specificity for the diagnosis of early cirrhosis. The presence or absence of liver cirrhosis in patients with chronic virus hepatitis can be detected using 2 or 3 quantitative and qualitative parameters, especially the length of spleen, the diameter of spleen vein and echo pattern of liver surface.

INTRODUCTION

Chronic hepatitis virus B or C infection results in damage to hepatocytes and may eventually lead to liver fibrosis, cirrhosis and/or hepatocellular carcinoma^[1-3]. The diagnosis of liver fibrosis and cirrhosis in patients with chronic virus hepatitis is of therapeutic and prognostic importance.

Although histologic examination of percutaneous biopsy specimens is the gold criterion for the severity of fibrosis and cirrhosis, biopsy is invasive and cannot be used repeatedly in follow-up. Moreover, liver biopsy can yield false negative results in nearly 20-30% of cases^[4-7]. Therefore, it is important to use noninvasive methods in differentiation between liver fibrosis and cirrhosis.

Ultrasonography (US) is a noninvasive and inexpensive procedure for diagnosis of focal and diffuse parenchymal disease of liver. Although US cannot detect minute changes, it can show liver cirrhosis in patients with decompensated liver function^[8-11]. However, correlation between US and histologic diagnosis has not been fully investigated in large series of patients. We conducted a prospective study to evaluate the validity of US for diagnosis of liver fibrosis in patients with chronic liver hepatitis without clinical or biochemical evidence of cirrhosis.

MATERIALS AND METHODS

Patients

From July 1999 to August 2002, 324 patients with chronic viral hepatitis undergoing US and histologic examination, were enrolled. Inclusion criteria included positive HBsAg and HBV-DNA or anti-HCV and HCV-RNA determined by PCR methods for at least 6 mo, and abnormal serum alanine transaminase level in recent 6 mo. Patients who had clinical or biochemical evidence of decompensated liver

Table 1 Relation between US quantitative parameters and fibrosis stages

| Parameters | Stage of fibrosis | Mean±SD | F | P |
|------------------------------|-------------------|------------|--------|--------|
| Length of spleen (cm) | S0 | 10.81±1.32 | 5.1947 | 0.0005 |
| | S1 | 10.82±1.45 | | |
| | S2 | 11.35±2.02 | | |
| | S3 | 13.31±2.68 | | |
| | S4 | 12.66±2.06 | | |
| Diameter of portal vein (cm) | S0 | 1.17±0.11 | 1.0369 | 0.3882 |
| | S1 | 1.18±0.17 | | |
| | S2 | 1.19±0.13 | | |
| | S3 | 1.20±0.12 | | |
| | S4 | 1.27±0.13 | | |
| Diameter of spleen vein (cm) | S0 | 0.62±0.16 | 6.7896 | 0.0000 |
| | S1 | 0.67±0.15 | | |
| | S2 | 0.72±0.18 | | |
| | S3 | 0.69±0.15 | | |
| | S4 | 0.81±0.20 | | |

function or portal hypertension, positive HIV antibody, serum titer of antinuclear antibody >1:160, serum creatinine level over 1.5 upper limit of normal value, or known liver diseases of other etiologies, were excluded.

Histologic examination

Percutaneous liver biopsy specimens were obtained from the anterior segment of the right lobe in each patient under the guidance of US using the quick-cut or Menghini biopsy needle. The satisfactory size of specimens was longer than 1 cm. The liver tissue samples were stained with hematoxylin-eosin, Gordon-Sweet and van-Gieson methods. Three pathologists performed the histological examination. To evaluate the inter-observer variation, Kappa analysis was conducted to control the quality of pathological diagnosis. The average Kappa value was 0.8144, indicating the excellent consistency for the staging of liver fibrosis.

According to the Guidelines of Prevention and Treatment of Viral Hepatitis of Chinese Medical Association (2000), liver fibrosis was divided into S0-S4 stages: S0 stage-no fibrosis, S1 stage-enlarged portal tracts with fiber proliferation, S2 stage-fibrosis of portal tract with formation of fiber septa and intact architecture of liver lobule, S3 stage-fibrosis with distortion of lobule architecture but without cirrhosis, S4 stage-definite cirrhosis. Liver inflammation was divided into grades from G1 (mild) to G4 (severe)^[12].

US examination

US examination was performed within 2 wk before or after liver biopsy. Acuson Aspen system with a 3.5-5.0 MHz curved probe was utilized. The two US operators were unaware of the clinical details and the results of biopsy. The results were recorded on video-tapes and the final report was given based on the consensus of both operators. The standard protocol of US examination included variables describing the liver size, surface and parenchyma, the ves-

sel structure, the blood flow velocity and spleen size.

Statistical analysis

Statistical analysis was performed using the SPSS 9.0 software. The significance of differences between subgroups was tested by the *F* test. $P < 0.05$ was considered statistically significant. A receive-operating characteristic (ROC) curve was used to determine the best cut-off values of US parameters for diagnosis of liver cirrhosis.

RESULTS

A total of 324 patients were collected, 272 men (83.9%) and 52 women (16.1%). Their age ranged from 18 to 60 years with a mean \pm SD of 35.56 ± 9.9 years. Based on virus markers, 306 patients had hepatitis B and 18 patients had hepatitis C. The mean duration of hepatitis, namely the duration from the date of clinical diagnosis to the date of liver biopsy, was 4.14 years. The histopathology showed S0 stage in 32 patients (9.9%), S1 stage in 116 patients (35.8%), S2 stage in 111 patients (34.3%), S3 stage in 35 patients (10.8%), S4 stage in 30 patients (9.3%), and inflammation G1 in 117 patients (36.1%), G2 in 110 patients (33.9%), G3 in 70 patients (21.6%) and G4 in 27 patients (8.3%).

The results of qualitative US were different in patients with various stages of liver fibrosis. The appearance of nodularities or irregular lines on liver surface and heterogenous distribution of nodularities in liver parenchyma, were related with advanced stages of liver fibrosis. But these descriptions could not definitely reflect the histopathologic diagnosis, because 97% patients in S0 subgroup and 66% patients in S4 subgroup showed smooth surface echo pattern. Only 13.7% patients in S4 stage subgroup showed moderate saw-teeth like liver surface echo pattern. No severe nodular surface was noted. The echo pattern of liver parenchyma and heterogenous distribution were significantly different in patients with various stages of liver fibrosis and were related to the severity of fibrosis. But coarse nodularity was frequently encountered: 25% in S0 subgroup patients, and 41% in S4 subgroup patients.

Table 1 summarizes the relation between liver fibrosis stages and quantitative US parameters, which showed the differences in subgroups with various stages of liver fibrosis. Among the quantitative US parameters, the spleen length and diameter of spleen vein were correlated with fibrosis stages ($P < 0.05$). The spleen lengths were significantly different in S1/S3, S2/S3, and S1/S4, but not significant in S3/S4 ($P = 0.43$). The diameter of spleen vein was significantly different in S2/S4 and S3/S4 ($P = 0.0068$ and $P = 0.0036$). However, the diameter of portal vein only increased significantly in patients with S4 stage of liver fibrosis. These data suggested that the length of spleen began to increase at S3 of liver fibrosis, so that the difference in S3/S4 was insignificant. The diameter of portal vein began to increase later than the length of spleen and diameter of spleen vein.

Based on receiver-operating characteristic (ROC) curve, the maximal sum of diagnostic sensitivity and specificity was considered as the best cut-off value of US parameters for prediction of the severity of liver fibrosis. The diag-

Table 2 Diagnostic values of three US parameters for early liver cirrhosis

| Parameters and cut-off value | Sensitivity | Specificity | Accuracy | Positive predicative value | Negative predicative value |
|---------------------------------|-------------|-------------|----------|----------------------------|----------------------------|
| Length of spleen (12.1 cm) | 0.600 | 0.753 | 0.737 | 0.198 | 0.948 |
| Diameter of spleen vein (8 mm) | 0.600 | 0.781 | 0.765 | 0.220 | 0.950 |
| Diameter of portal vein (12 mm) | 0.767 | 0.446 | 0.475 | 0.124 | 0.949 |

nostic values of three quantitative US parameters are listed in Table 2.

DISCUSSION

Liver cirrhosis can be detected by US in patients with portal hypertension. Schalm^[13] reviewed the diagnostic methodology of liver cirrhosis and found that percutaneous liver biopsy has a sensitivity of below 85% in detection of liver cirrhosis. Liver biopsy could yield false negative results in nearly one third of cases, but it is currently considered the criterion for establishing a precise diagnosis and assessing the extent of fibrosis. The diagnostic sensitivity and specificity of US examination for liver cirrhosis vary widely with a diagnostic sensitivity of 0.125- 0.95 and a diagnostic specificity of 0.285- 1.0^[14-16]. The diagnostic accuracy of US for early liver cirrhosis in patients with chronic virus hepatitis and compensated liver function has not been fully investigated.

At US scanning, liver surface nodularity reflects the presence of regenerative nodules and fibrous septa. Nodularity on liver surface and in parenchyma is independently associated with the diagnosis of cirrhosis and US is reliable for diagnosis of liver fibrosis. It was reported that the high frequency US transducer (7.5-12 MHz) can obtain satisfactory results for diagnosis of liver cirrhosis^[17] while the low frequency US is not a reliable test for liver cirrhosis^[18].

Gaiani *et al*^[19] showed that 80.4% of cirrhosis can be detected in patients with compensated liver diseases of various etiologies using a US scoring system based on two US parameters. Colli *et al*^[20] reported that US can detect severe fibrosis or cirrhosis with a specificity of 0.95 and a sensitivity of only 0.54. Moreover, surface nodularity could also be influenced by different factors, mainly local fatty infiltration. Hung *et al*^[21] evaluated the validity of US in diagnosis of cirrhosis with a diagnostic sensitivity of liver cirrhosis of 0.775 and a specificity of 0.92 in patients with HBV infection. Zheng *et al*^[22] studied the value of US in evaluation of liver fibrosis and compensated cirrhosis in comparison with serology and histology and found that hepatic parenchymal echo pattern, liver surface and thickness of gallbladder wall are three independent predictors of liver fibrosis. The diagnostic accuracy of US for compensated cirrhosis is 80.7%.

The accuracy of Doppler US measurement for diagnosis of early liver cirrhosis is still controversial. It was reported that decreased flow velocity in portal vein is sufficiently accurate in diagnosis of liver cirrhosis^[23-25]. While other studies^[26-28] showed that substantial variability exists in measurement of portal venous blood flow velocity and volume. Doppler US measurement does not represent the hepatic venous pressure gradient^[29]. This controversy could

be explained by the lack of standard technique of Doppler measurement. Furthermore, changes of hemodynamics in hepatic blood flow are influenced by multiple factors, such as extent of fibrosis, chronic inflammation, presence and size of esophageal varices, as well as porto-systemic shunts.

The results of this study showed that the maximal velocity of blood flow in portal vein was weakly related with liver cirrhosis, but the standard deviation of data was wide, suggesting that this Doppler US parameter is not important in evaluation of liver fibrosis.

Schalm^[13] suggested that if histology shows no cirrhosis (nodules surrounded by fibrosis) but fibrosis and architectural distortion, diagnosis of cirrhosis should still be made when there is a US diagnosis of cirrhosis. Afdhal and Nunes^[30] argued that a proper US examination can identify patients with cirrhosis when the biopsy findings are equivocal, or at variance with the clinical impression.

The results of this study showed that different stages of hepatic fibrosis could not be determined satisfactorily by US parameters. A single US parameter was limited in sensitivity and specificity for diagnosis of early cirrhosis. Early liver cirrhosis could be excluded using two or three quantitative and qualitative US parameters, especially the spleen length, diameter of spleen vein and echo pattern of liver surface, because the negative predictive values of these three quantitative US parameters were high. US can also be used in follow-up of patients with chronic virus hepatitis.

The limitations of our study are the relatively small number of patients with S0- S4 stages of liver fibrosis and no application of the high-frequency US probe in examination of liver surface.

In conclusion, US cannot be used as a specific diagnostic tool for chronic viral hepatitis, but US should be stressed in screening and follow-up of patients with chronic virus hepatitis. However, the results of this study do not decrease the value of liver biopsy because it has other indications in clinical practice of hepatology.

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RAPID COMMUNICATION

Clinicopathologic characteristics of esophagectomy for esophageal carcinoma in elderly patients

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Abstract

AIM: To evaluate the risk of esophagectomy for carcinoma of the esophagus in the elderly (70 years or more) compared with younger patients (<70 years) and to determine whether the short-term outcomes of esophagectomy in the elderly have improved in recent years.

METHODS: Preoperative risks, postoperative morbidity and mortality in 60 elderly patients (≥ 70 years) with esophagectomy for carcinoma of the esophagus were compared with the findings in 1 782 younger patients (<70 years) with esophagectomy between January 1990 and December 2004. Changes in perioperative outcome and short-time survival in elderly patients between 1990 to 1997 and 1998 to 2004 were separately analyzed.

RESULTS: Preoperatively, there were significantly more patients with hypertension, pulmonary dysfunction, cardiac disease, and diabetes mellitus in the elderly patients as compared with the younger patients. No significant difference was found regarding the operation time, blood loss, organs in reconstruction and anastomotic site between the two groups, but elderly patients were more often to receive blood transfusion than younger patients. Significantly more transhiatal and fewer transthoracic esophagectomies were performed in the elderly patients as compared with the younger patients. Resection was considered curative in 71.66% (43/60) elderly and 64.92% (1 157/1 782) younger patients, which was not statistically significant ($P > 0.05$). There were no significant differences in the prevalence of surgical complications between the two groups. Postoperative cardiopulmonary medical complications were encountered more frequently in elderly patients. The hospital mortality rate was 3.3% (2/60) for elderly patients and 1.1% (19/1 782) for younger patients without a significant difference. When the study period was divided into a former (1990 to 1997) and a recent (1997 to 2004) period,

operation time, blood loss, and percentage of patients receiving blood transfusion of the elderly patients significantly improved from the former period to the recent period. The hospital mortality rate of the elderly patients dropped from the former period (5.9%) to the recent period (2.3%), but it was not statistically significant.

CONCLUSION: Preoperative medical risk factors and postoperative cardiopulmonary complications after esophagectomy are more common in the elderly, but operative mortality is comparable to that of younger patients. These encouraging results and improvements in postoperative mortality and morbidity of the elderly patients in recent period are attributed to better surgical techniques and more intensive perioperative care in the elderly.

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Key words: Esophagectomy; Carcinoma; Esophagus

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INTRODUCTION

Perioperative management of elderly patients after a major operation is an important issue because of the recent worldwide increase in the elderly population. Esophagectomy for esophageal carcinoma is a major procedure associated with a high mortality and morbidity, and advanced age is often considered a significant risk factor and even a relative contraindication to esophagectomy despite advances in modern surgical practice^[1-3].

There have been a small number of studies on the relationship between the clinicopathologic characteristics and age of patients after esophagectomy for esophageal carcinoma. However, whether the prognosis of elderly patients after esophagectomy is more unfavorable than that in younger patients remains still controversial. Some reports emphasized the worse prognosis in elderly patients after esophagectomy^[4,5], whereas others emphasized similar outcome irrespective of the age^[6-8].

Table 1 Clinicopathologic characteristics *n* (%)

| Variables | ≥70 years (<i>n</i> =60) | < 70 years (<i>n</i> =1782) | <i>P</i> value |
|--------------------------------|------------------------------|---------------------------------|----------------|
| Mean age (yr) | 73.1±3.9 | 55.8±5.2 | 0.0007 |
| Sex (male/female) | Sep-51 | 1453/329 | 0.61 |
| Tumor location | | | 0.74 |
| Cervical | 3 (5.0) | 75 (4.2) | |
| Upper-third | 7 (11.7) | 186 (10.4) | |
| Middle-third | 32 (53.3) | 901 (50.6) | |
| Lower-third | 16 (26.7) | 591 (33.2) | |
| Double location | 2 (3.3) | 29 (1.6) | |
| Tumor size (cm) | 5.2±2.1 | 6.5±2.7 | 0.85 |
| Histological type | | | 0.87 |
| Squamous cell carcinoma | 53 (88.3) | 1 610 (90.3) | |
| Adenocarcinoma | 4 (6.7) | 97 (5.4) | |
| Other carcinomas | 3 (5.0) | 75 (4.2) | |
| Histological differentiation | | | 0.76 |
| Well | 8 (13.3) | 185 (10.4) | |
| Moderately | 46 (76.7) | 1 410 (79.1) | |
| Poorly | 6 (10.0) | 187 (10.5) | |
| TNM stage | | | 0.89 |
| 0 | 1 (1.7) | 15 (0.8) | |
| I | 2 (3.3) | 94 (5.3) | |
| II | 9 (15.0) | 251 (14.1) | |
| III | 45 (75.0) | 1 308 (73.4) | |
| IV | 3 (5.0) | 114 (6.4) | |
| Preoperative radiochemotherapy | 19 (31.7) | 624 (35.0) | 0.68 |

The purpose of this study was to evaluate the risk of esophagectomy for the esophageal carcinoma in the elderly (70 years or more) as compared with younger patients (<70 years) and to determine whether the short-term outcome of esophagectomy in the elderly have improved with increased experience of the surgical team and improved perioperative management in recent years.

MATERIALS AND METHODS

Patients

The subjects included 1 842 consecutive patients with primary carcinoma of the esophagus, who had been treated by esophageal resection and reconstruction between January 1990 and December 2004 in our institute. Our patients were unselected and consisted of all of the patients after esophagectomy for esophageal carcinoma during the study period. The patients comprised 1 563 men and 279 women. Sixty were elderly (≥70 years) and 1 782 were younger patients (<70 years). Among the elderly, 17 patients were operated during the period 1990 to 1997, and data obtained were compared with those of 43 patients operated during the period 1998 to 2004.

All patients had detailed preoperative risk assessments based on history of chronic lung or heart disease, chest x-ray, electrocardiogram (ECG), arterial blood gas analysis, pulmonary function tests, and biochemical and hematological tests. The preoperative risk factors analyzed included weight loss more than 10%, anemia (hemoglobin less than 12 g/L), hypertension (prescribed history of hypertension, systolic blood pressure more than 140 mm Hg, and/or diastolic pressure more than 90 mm Hg), chronic pulmonary disease or abnormal lung dysfunction (forced expiratory volume at 1 second [FEV₁] < 70%

Table 2 Preoperative risks *n* (%)

| Risks | ≥70 years (<i>n</i> =60) | < 70 years (<i>n</i> =1 782) | <i>P</i> value |
|-----------------------|------------------------------|----------------------------------|----------------|
| Weight loss | 17 (28.3) | 564 (31.6) | 0.67 |
| Anemia | 13 (21.7) | 344 (19.3) | 0.62 |
| Hypertension | 26 (43.3) | 512 (28.7) | 0.02 |
| Pulmonary dysfunction | 43 (71.7) | 479 (26.9) | 0 |
| Cardiac disease | 23 (38.3) | 324 (18.2) | 0 |
| Cirrhosis | 8 (13.3) | 155 (8.7) | 0.24 |
| Chronic renal disease | 11 (18.3) | 236 (13.2) | 0.25 |
| Diabetes mellitus | 24 (40.0) | 395 (22.2) | 0.003 |

of predicted normal), cardiac disease (history of ischemic heart disease, heart failure, or abnormal ECG), cirrhosis, chronic renal disease, and diabetes mellitus. Clinicopathologic characteristics, therapeutic methods, and the postoperative morbidity and mortality between elderly and younger patients were compared. Hospital mortality was defined as death within the same hospital admission after surgery, up to 6 mo after surgery. Resection was defined as curative when the tumor was confined to the esophagus with or without involvement of adjacent lymph nodes and all macroscopic tumors had been removed. Resection was palliative when there was infiltration of the tumor beyond the esophagus into mediastinal organs or when there was residual tumor after resection.

Statistical analysis

Comparisons between groups were performed using the Student's *t* test and χ^2 test or Fisher's exact test. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

Clinicopathologic characteristics and preoperative risks

The clinicopathologic characteristics between the elderly and younger groups are shown in Table 1. There were no significant differences in sex, tumor location, tumor size, histological type, histological differentiation, TNM stage and preoperative radiochemotherapy between the two groups. There were significantly more patients with hypertension, pulmonary dysfunction, cardiac disease, and diabetes mellitus in the elderly group, whereas weight loss, anemia, cirrhosis and chronic renal disease status were not statistically different between the two groups (Table 2).

Surgical treatment

Operative variables are shown in Table 3. No significant difference was found regarding the operation time, blood loss, organs in reconstruction and anastomotic site between the two groups, but elderly patients were more often to receive blood transfusion than younger patients. Significantly more transhiatal and fewer transthoracic esophagectomies were performed in the elderly patients. Resection was considered curative in 71.66% (43/60) elderly and 64.92% (1 157/1 782) younger patients, which difference was not significant (*P* > 0.05).

Table 3 Surgical treatment *n* (%)

| Variables | ≥70 years (<i>n</i> = 60) | < 70 years (<i>n</i> = 1782) | <i>P</i> value |
|-----------------------------|-------------------------------|----------------------------------|----------------|
| Operation time (min) | 239±147 | 225±139 | 0.88 |
| Blood loss (mL) | 443±364 | 418±251 | 0.41 |
| Blood transfusion | 23 (38.3) | 415 (23.3) | 0.01 |
| Types of operations | | | 0 |
| Transthoracic esophagectomy | 41 (68.3) | 1 563 (87.7) | |
| Transhiatal esophagectomy | 17 (28.3) | 190 (10.7) | |
| Thoracoscopic esophagectomy | 2 (3.3) | 29 (1.6) | |
| Organs in reconstruction | | | 0.27 |
| Stomach | 53 (88.3) | 1 435 (80.5) | |
| Colon | 3 (5.0) | 197 (11.1) | |
| Jejunum | 4 (6.7) | 150 (8.4) | |
| Anastomotic site | | | 0.31 |
| Cervical | 14 (23.3) | 326 (18.3) | |
| Intrathoracic | 46 (76.7) | 1 456 (81.7) | |
| Curative resection | 43 (71.7) | 1 157 (64.9) | 0.34 |

Table 4 Postoperative morbidity and mortality *n* (%)

| Variables | ≥70 years (<i>n</i> = 60) | < 70 years (<i>n</i> = 1782) | <i>P</i> value |
|---------------------------|-------------------------------|----------------------------------|----------------|
| Surgical complications | | | |
| Anastomotic leakage | 2 (3.3) | 35 (2.0) | 0.34 |
| Hemorrhage | 1 (1.7) | 19 (1.0) | 0.49 |
| Intra-abdominal abscess | 0 (0.0) | 8 (0.4) | 1 |
| Chylothorax | 2 (3.3) | 39 (2.2) | 0.39 |
| Thoracic empyema | 0 (0.0) | 12 (0.7) | 1 |
| Recurrent nerve paralysis | 1 (1.7) | 20 (1.1) | 0.5 |
| Wound dehiscence | 1 (1.7) | 23 (1.3) | 0.55 |
| Medical complications | | | |
| Pulmonary | 26 (43.3) | 501 (28.1) | 0.01 |
| Cardiac | 23 (38.3) | 352 (19.8) | 0.001 |
| Renal | 1 (1.7) | 46 (2.6) | 1 |
| Hepatic | 0 (0.0) | 15 (0.8) | 1 |
| Postoperative deaths | 2 (3.3) | 19 (1.1) | 0.15 |
| Anastomotic leakage | 1 (1.7) | 8 (0.4) | 0.26 |
| Pulmonary disease | 1 (1.7) | 9 (0.5) | 0.28 |
| Cerebrovascular accident | 0 (0.0) | 2 (0.1) | 1 |

Postoperative morbidity and mortality

Postoperative morbidity and mortality in the elderly and younger groups are shown in Table 4. There were no significant differences in the prevalence of surgical complications between the two groups. Although the anastomotic leakage rate was low, it was still the most common surgical complication in each group. Postoperative medical complications were encountered more frequently in the elderly, mainly pulmonary and cardiac, whereas other medical complications were not statistically different between the two groups. No patient died on the operation table. The hospital mortality rate was 3.3% (2/60) for elderly patients and 1.1% (19/1 782) for younger patients without a significant difference. There was no significant difference in the types of complication as the causes of death between the two groups.

When the study period was divided into a former (1990 to 1997) and a recent (1997 to 2004) period, operative time, blood loss, and percentage of patients receiving blood transfusion of the elderly patients significantly improved from the former period to the recent period

Table 5 Postoperative morbidity and mortality in elderly patients during the two periods *n* (%)

| Variables | 1990-1997 (<i>n</i> = 17) | 1998-2004 (<i>n</i> = 43) | <i>P</i> value |
|---------------------------|-------------------------------|-------------------------------|----------------|
| Operation time (min) | 255±157 | 212±104 | 0.008 |
| Blood loss (mL) | 520±375 | 402±249 | 0.003 |
| Blood transfusion | 13 (76.4) | 19 (44.2) | 0.04 |
| Surgical complications | | | |
| Anastomotic leakage | 1 (5.9) | 1 (2.3) | 0.49 |
| Hemorrhage | 0 (0.0) | 1 (2.3) | 1 |
| Chylothorax | 1 (5.9) | 1 (2.3) | 0.49 |
| Recurrent nerve paralysis | 1 (5.9) | 0 (0.0) | 1 |
| Wound dehiscence | 0 (0.0) | 1 (2.3) | 1 |
| Medical complications | | | |
| Pulmonary | 11 (64.7) | 15 (34.9) | 0.046 |
| Cardiac | 10 (58.8) | 13 (30.2) | 0.04 |
| Renal | 0 (0.0) | 1 (2.3) | 1 |
| Hospital mortality rate | 1 (5.9) | 1 (2.3) | 0.49 |

(Table 5). No significant differences in surgical complications were observed, but there was a significant decrease in postoperative cardiopulmonary complications from the former period to the recent period. The hospital mortality rate of the elderly patients dropped from the former period (5.9%) to the recent period (2.3%), but it was not statistically significant.

DISCUSSION

Esophageal carcinoma in the elderly has increased in part because of increasing life expectancy. Esophagectomy for carcinoma probably has the highest operative mortality of any elective surgical procedures^[9]. Therefore, it is important to evaluate the risk of esophagectomy for carcinoma in elderly patients. Advanced age was once considered a relative contraindication to esophagectomy because of the high operative mortality rate^[4,5]. At the same time, the malignant potential of neoplasms in elderly patients has occasionally been reported to be much less aggressive than that in younger patients^[10]. Whether the prognosis of elderly patients with esophagectomy for esophageal carcinoma is more unfavorable than that in younger patients remains controversial.

Recently, more and more reports emphasized that esophagectomy could be performed in a high percentage of elderly patients and thus advanced age alone should not be considered as a contraindication for esophagectomy^[6-8]. We shared this opinion and believed that resection should be offered whenever possible because it offered the only hope of cure and the best method of palliation^[11]. The presence of risk factors has great impact on surgical outcome, hence thorough preoperative assessment should be carried out in all patients. Even in the presence of medical risk factors, resection is still preferred for the elderly unless the risk is prohibitively high. Most of our patients who did not have resection were unresectable because of extensive local or metastatic disease, and only a small portion (about 20%) of elderly patients were deemed unresectable because of poor physical conditions or cardiopulmonary status.

Preoperative risk assessment is an important aspect of patient selection for esophagectomy, as a significant number of these patients had cardiopulmonary or diabetes mellitus in the preoperative period and cardiopulmonary complications in the postoperative period. Pulmonary complication was one of the most common causes of surgical complication-related deaths in both groups. These results strongly suggest that greater preoperative precautions must be taken to manage cardiopulmonary complications in the elderly patients^[12]. The anastomotic leakage and chylothorax rate were low in both elderly and younger patients, but these remained the main surgical complications. Pulmonary complication was the most common cause of postoperative death in both elderly and younger patients.

Although a significant number of patients had postoperative surgical or medical complications, only a few of them succumbed to death because of those complications. The mortality rate caused by surgical or medical complications in elderly patients was slightly higher but comparable to that of younger patients, despite higher cardiopulmonary risk and more cardiopulmonary complications in the elderly. The similar outcome was probably the result of significant improvement in surgical technique and more intensive perioperative patient care in our institution. For example, chest physiotherapy was instituted early before operation, and during the postoperative period, cricothyroidotomy was often performed to keep the airway clear of sputum. The more frequent use of transthiatal over transthoracic esophagectomy in elderly patients may also have contributed to the low cardiopulmonary-related mortality^[13]. Moreover, shorter operative time, reduced blood loss and fewer perioperative blood transfusion in recent years may all have important impact on the reduced incidence of cardiopulmonary complications during this period^[14-17]. Finally, there has been considerable improvement in postoperative pain control by epidural anesthesia block. Adequate analgesia decreases pulmonary complications by decreasing the disturbances of pulmonary mechanics after thoracotomy or laparotomy and enabling patients to generate effective cough^[18]. The pulmonary complication rate decreased to only 34.9% in recent years in contrast to 64.7% in the previous era.

In conclusion, our study showed that preoperative medical risk factors and postoperative cardiopulmonary complications after esophagectomy are more common in the elderly, but operative mortality was comparable to that of younger patients. These encouraging results and improvements in postoperative mortality and morbidity in recent period are attributed to better surgical techniques and more intensive perioperative care in elderly patients. However, a careful patient selection procedure must be used to exclude the high-risk elderly patients from the operative list and thus will help to reduce the postoperative morbidity and mortality rate in this group of patients.

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RAPID COMMUNICATION

Value of mink vomit model in study of anti-emetic drugs

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Abstract

AIM: To establish a new, reliable vomit model of minks.

METHODS: Adult male minks were randomly divided into 8 groups ($n=6$): cisplatin (7.5 mg/kg) intraperitoneal injection (ip) group, copper sulfate (40 mg/kg) intragastric injection (ig) group, apomorphine (1.6 mg/kg) subcutaneous injection (sc) group, and 18 Gy whole-body X-irradiation group, ondansetron injection group (2 mg/kg ip) 30 min later followed by cisplatin (7.5 mg/kg) ip, normal saline (NS) ip injection control group, metoclopramide injection group (4 mg/kg ip) 30 min later followed by apomorphine (1.6 mg/kg) sc, NS ig control group. The frequency of retching and vomiting was calculated. After behavioral experiment, distribution of 5-HT in the ileum was detected by immunohistologic method.

RESULTS: Cisplatin, apomorphine, copper sulfate and X-irradiation administered to minks evoked a profound emetic response in the animals. However, retching and vomiting were significantly inhibited by pretreatment with ondansetron and metoclopramide in cisplatin and copper sulfate groups ($P=0.018$). Immunohistologic result showed that 5-HT released from enterochromaffin cells (EC cells) was involved in vomiting mechanism.

CONCLUSION: Mink vomit model has a great value in studying the vomiting mechanism and screening new antiemetic drugs.

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Key words: Vomit; Mink; Cisplatin; Ondansetron; Apomorphine; X-irradiation

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INTRODUCTION

In the treatment of malignant disease, potentially curative chemotherapy and radiotherapy are commonly associated with a wide range of adverse events, including intractable nausea and vomiting, which can challenge patient compliance with a treatment regimen. Other forms of emesis, including those experienced post-operatively or in other diseases, can also present as serious problems. Studying the vomiting mechanism and screening for new anti-emetic drugs urge us to establish appropriate animal models with similar vomiting behavior to that of human beings. Ferret (*Mustela putorius furo*) is a relative ideal animal model in vomiting study worldwide^[1]. However, its use is limited because of its high feeding cost and difficulties to survive. Fortunately minks (*Mustela vison*) and ferrets belonging to the same stoat genus are characterized by their inexpensiveness, wide availability and ease of feeding and raising. However, there are no reports on the use of minks in medical researches. This study was to establish a new and reliable vomit model of minks based on the effects of a variety of emetogens.

MATERIALS AND METHODS

Materials

Cisplatin powder was obtained from Qilu Pharmaceutical Factory, Jinan, China. Ondansetron hydrochloride injection was from Ningbo Tianheng Pharmaceutical Factory, Ningbo, China. Copper sulfate was from Shanghai Hengda Chemical Co., Shanghai, China. Apomorphine was from Sigma in St Louis, USA. Metoclopramide dihydrochloride injection was from Tianjin People's Pharmaceutical Factory, Tianjin, China. SABC immunohistology test kit was from Boster Biotechnology Co., Wuhan, China. Varian 6 MeV 2100-C X-irradiation Linear Accelerator was from Varian Corp., USA. Microscope was from Olympus, Japan. Adult male minks weighing 1.3-1.8 kg were provided by Qingdao Special Animal Center.

Animal experiments

Animals were used in the present study in accordance with the Qingdao University Guide for the Care and Use of Laboratory Animals.

To examine the effects of emetogens, the minks were divided into 4 groups ($n=6$), and housed individually in an iron cage of 75 cm × 50 cm × 50 cm with free access to

Table 1 Effects of emetogens on minks ($n = 6$, mean \pm SD)

| Emetogens | Dose (mg/kg) | Latency (min) | Retching (n) | Emesis (n) | Vomiting ratio |
|--------------------|--------------|---------------|---------------|---------------|----------------|
| NS | - | - | 0 | 0 | 0/6 |
| Cisplatin | 7.5 | 92.0 \pm 21 | 91.5 \pm 37 | 12.0 \pm 3 | 6/6 |
| NS | - | - | 0 | 0 | 0/6 |
| Apomorphine | 1.6 | 39.4 \pm 36 | 28.5 \pm 16 | 5.3 \pm 1.9 | 6/6 |
| NS | - | - | 0 | 0 | 0/6 |
| Copper sulfate | 40 | 7.0 \pm 2.0 | 29.0 \pm 9 | 8.7 \pm 2.3 | 6/6 |
| Pseudo irradiation | 0 | - | 0 | 0 | 0/6 |
| X-ray | 18 Gy | 17.0 \pm 4 | 19.3 \pm 7 | 10.4 \pm 3 | 5/6 |

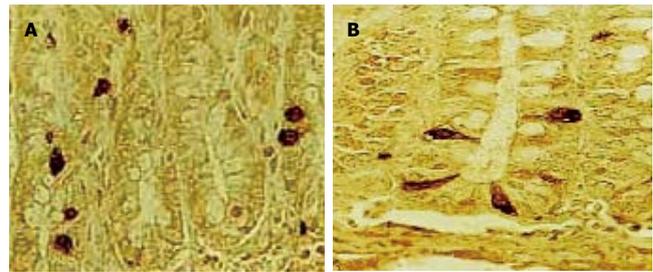


Figure 1 A: control group, 5-HT did not release from EC cells with a clear round rim; B: the cisplatin group, 5-HT released from EC cells with a tail.

Table 2 Effects of ondansetron or metoclopramide on emesis induced by cisplatin or apomorphine in minks (mean \pm SD, $n = 6$)

| Emetogens | Dose (mg/kg) | Anti-emetics | Dose (mg/kg) | Latency (min) | Retching (n) | Emesis (n) |
|-------------|--------------|----------------|--------------|----------------|--------------------------|----------------------------|
| Cisplatin | 7.5 | NS | - | 92.0 \pm 21 | 91.5 \pm 37 | 12 \pm 3 |
| Cisplatin | 7.5 | Ondansetron | 2 | 143.8 \pm 80 | 47.3 \pm 41 | 5.3 \pm 5 ^a |
| Apomorphine | 1.6 | NS | - | 39.4 \pm 36 | 28.5 \pm 16 | 5.3 \pm 1.9 |
| Apomorphine | 1.6 | Metoclopramide | 4 | -a | 1.3 \pm 3 ^b | 0.3 \pm 0.8 ^d |

^a $P=0.018$, $t=2.93$ vs vehicle-pretreated cisplatin group; ^b $P=0.0022$, $t'=4.08$, ^d $P=0.003$, $t'=5.94$ vs vehicle-pretreated apomorphine group; -a: only 1 monk had retching and vomiting.

food and water

The minks in the first group were first given NS (5 mL/kg ip). After 24 h, the animals were administered cisplatin (7.5 mg/kg ip) in a volume of 5 mL/kg diluted with NS.

The minks in the second group were first given NS (15 mL/kg ig). After 24 h, the animals were given copper sulfate (40 mg/kg ig) in a volume of 15 mL/kg diluted with NS.

The minks in the third group were first given NS (0.4 mL/kg sc). After 24 h, the animals were given apomorphine (1.6 mg/kg sc) in a volume of 0.4 mL/kg diluted with NS.

The minks in the fourth group were first exposed to pseudo-irradiation. After 24 h, the animals received 18 Gy whole-body X-irradiation for 4.5 min.

To investigate the effects of anti-emetic agents on emesis induced by cisplatin or apomorphine, the minks in the first group were injected intraperitoneally with ondansetron injection (2 mg/kg) in a volume of 1 mL/kg 30 min before the intraperitoneal administration of cisplatin (7.5 mg/kg).

The minks in the second group were pretreated with NS (1 mL/kg ip) as control. After 30 min, the animals were administered cisplatin (7.5 mg/kg).

The minks in the third group were pretreated with metoclopramide injection (4 mg/kg ip) in a volume of 0.8 mL/kg, followed by apomorphine (1.6 mg/kg sc) after 30 min.

The minks in the fourth group were pretreated with NS (0.8 mL/kg ip) as control, followed by apomorphine (1.6 mg/kg sc) later 30 min.

All experiments were carried out starting at 9:00 am. After treatment, retching and vomiting were observed for 6 h, following the vomit criteria for ferrets described by Minami *et al.*^[2]. No observers were aware of the treatments. Salivating, flinching and other behaviors resembling the nausea of human beings were observed. During vomiting,

the mink's head was protruding downwards ahead with open mouth, shrugging shoulder, contracting abdomen and occasional sound of vomiting. A vomiting cycle started the minute when vomiting began until smooth breaths recovered. A retching/vomiting referred to a vomiting action without or with stomach content spat out. The frequency of retching and vomiting was calculated.

Immunohistologic analysis

Minks in control group ($n=3$) and cisplatin group ($n=3$, 3 h after 7.5 mg/kg ip) were killed by cervical dislocation. A 3 cm long section was dissected 20 cm from the pylorus, and the distribution of 5-HT was detected under microscope by the immunohistologic method according to the instructions of the SABC test kit.

Statistical analysis

Data were expressed as mean \pm SD and analyzed by Student's t test. $P < 0.05$ was considered statistically significant.

RESULTS

Animal experiments

Cisplatin, apomorphine, copper sulfate and X-irradiation administered to the minks evoked a profound emetic response, and the manifestations of retching and vomiting in minks were similar to those in ferrets^[2]. No vomiting occurred in control groups. The effects of emetogens over a 6 h period are shown in Table 1.

All minks in the NS + cisplatin group suffered from retching and vomiting. Vomiting started after 60 min and reached its peak after about 2 h. Five minks in the ondansetron pretreatment group suffered from retching and vomiting. The frequency of cisplatin-induced retching and vomiting after 6 h was significantly reduced by pretreatment with ondansetron.

All minks in the NS+apomorphine group and only one in the metoclopramide pretreatment group, suffered from retching and vomiting. The frequency of apomorphine-induced retching and vomiting after 6 h was significantly reduced by pretreatment with metoclopramide.

No significant difference was found between the pretreatment and control groups in terms of the latency and duration of emesis induced by cisplatin or apomorphine (Table 2).

Immunohistologic analysis

In the cisplatin group, 5-HT was released from EC cells which seemed to have a tail. In the control group, 5-HT was not released from EC cells which was round with a clear rim. Similar phenomena were also observed in other groups (Figure 1).

DISCUSSION

It is widely known that vomiting cannot be induced in rodents such as mice (except for the special strains), rats, and guinea pigs. Pigeons, cats and dogs are occasionally used in vomiting research and screening for new anti-emetics. Since pigeons often carry infectious viruses to human beings, no reports are available on vomiting induced by anticancer drugs in pigeons. Cats and dogs have a good learning ability. When vomiting is induced by emetogens, it often continues even anti-emetics^[3] are administered. Ferret is a widely accepted animal model in vomiting study at present, mainly because its vomiting behavior resembles that of human beings^[4] in terms of behavior, responses to emetogens and anti-emetic agents, and biochemistry change in vomit response. Ondansetron, a potential anti-emetic agent, was developed in ferret vomit model by Glaxo Company. However, since ferret is so expensive and difficult to raise, its wide use cannot be realized. In fact, no laboratory has carried out experiments in ferrets so far in China. Minks belong to the same stoat family of ferrets. As a vomit model, they not only share

the advantages with ferrets, but also are inexpensive and can be raised quite easily and widely.

In the present study, the effects of a variety of emetogens, including cisplatin, copper sulfate, apomorphine and X-irradiation at the doses evoking a profound emetic response in ferrets, were identical in minks. The manifestations of retching and vomiting were similar to those in ferrets. As shown in our experiment, ondansetron and metoclopramide can inhibit emesis induced by cisplatin and apomorphine in minks.

5-HT immunohistologic analysis showed that emetogens increased the release of 5-HT from EC cells in intestine, which may be an important mechanism involved in vomiting. This finding is consistent with the results reported previously^[5]. However, it must be acknowledged that this study has its limitations because our experiments were mainly focused on the behavior of minks and the vomiting mechanism was studied in the initial stage. A further study is needed.

In conclusion, the mink vomit model is of great value in studying the vomiting mechanism and screening for new anti-emetic drugs.

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Arg-gly-asp-mannose-6-phosphate inhibits activation and proliferation of hepatic stellate cells *in vitro*

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Abstract

AIM: To investigate the effect of arg-gly-asp-mannose-6-phosphate (RGD-M6P) on the activation and proliferation of primary hepatic stellate cells *in vitro*.

METHODS: Hepatic stellate cells (HSCs) were isolated from rats by *in situ* collagenase perfusion of liver and 18% Nycodenz gradient centrifugation and cultured on uncoated plastic plates for 24 h with DMEM containing 10% fetal bovine serum (FBS/DMEM) before the culture medium was substituted with 2% FBS/DMEM for another 24 h. Then, HSCs were cultured in 2% FBS/DMEM with transforming growth factor β 1, M6P, RGD, or RGD-M6P, respectively. Cell morphology was observed under inverted microscope, smooth muscle α -actin (α -SMA) was detected by immunocytochemistry, type III procollagen (PCIII) in supernatant was determined by radioimmunoassay, and the proliferation rate of HSCs was assessed by flow cytometry.

RESULTS: RGD-M6P significantly inhibited the morphological transformation and the α -SMA and PC III expressions of HSCs *in vitro* and also dramatically prevented the proliferation of HSCs *in vitro*. Such effects were remarkably different from those of RGD or M6P.

CONCLUSION: The new compound, RGD-M6P, which has a dramatic effect on primary cultured HSCs *in vitro*, can inhibit the transformation of HSCs in culture caused by TGF β 1, suppresses the expression of PCIII and decreases proliferation rate of HSC. RGD-M6P can be applied as a selective drug carrier targeting at HSCs, which may be a new approach to the prevention and treatment of liver fibrosis.

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Key words: RGD; Mannose-6-phosphate; Hepatic stellate

INTRODUCTION

Liver fibrosis is characterized by an excessive deposition of extracellular matrix constituents, resulting from an enhanced synthesis of matrix proteins (fibrogenesis) and decreased removal by matrix degrading enzymes (fibrolysis) in consequence of liver cell damage of various causes^[1-3]. The major cell type responsible for hepatic fibrogenesis is the activated hepatic stellate cells (HSCs). Therefore, this cell type is an important target for antifibrotic therapy^[4,5]. Pharmacotherapeutic intervention to alter HSC functions may act at different levels: inhibition of activation and transformation of HSCs, inactivation of profibrogenic cytokines, interference with matrix synthesis and stimulation of matrix degradation^[6-11]. *In vivo*, however, antifibrotic drugs may not be efficiently taken up by HSCs or may produce undesirable side effects. Drug targeting may be explored to elicit cell-specific uptake of drugs, but in contrast to other cell types in the liver, a specific carrier agent to go into HSCs is not yet known^[12]. Therefore, we focused on the development of a specific carrier for HSCs. The binding sites expressed on activated HSCs were considered for their ability to serve as potential targets for carrier molecules. Two of these are integrin and mannose 6-phosphate/insulin like growth factor II (M6P/IGF-II) receptors, which are over-expressed on activated rat HSCs, particularly during fibrosis^[13,14]. Additionally, extracellular matrixes (ECM) regulate the morphology and biological activities of HSCs through integrins on the cell membrane^[13]. It has been certified that the RGD sequence plays a vital role in the linkage between ECM and integrins^[13]. This study was to synthesize a new compound, RGD-M6P targeting at integrins and/or M6P/IGF-II receptors over-expressed on activated HSCs and to study its effects on the activation and proliferation of primary cultured HSCs in order to find a new approach for regulating HSC activation and anti-hepato-fibrotic therapy. If successful, this may be of value in the design of other receptor-specific carriers.

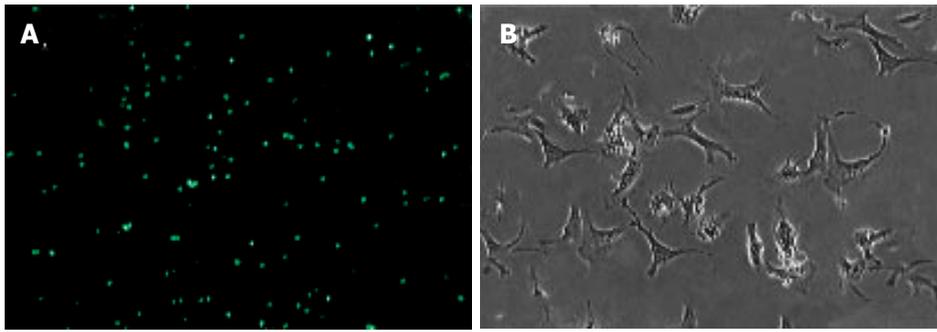


Figure 1 Small and round HSCs in the presence of vitamin-A droplets in cytoplasm (A) and spindle-like or asteroid membranous processes in HSCs (B).

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 400-500 g were purchased from the Shanghai Laboratory Animal Center, Chinese Academy of Sciences.

Main reagents

RGD, M6P and RGD-M6P were synthesized in Gastroenterological Laboratory of Xinhua Hospital, Shanghai Second Medical University. DMEM was purchased from Gibco Company. Procollagen III (PCIII) radioimmunoassay kits were purchased from Institute of Naval Medicine, Shanghai. α -SMA monoclonal antibody was provided from by Cruz Company.

Isolation of HSCs and grouping

HSCs were isolated and cultured. Briefly, the rats were anesthetized with an intraperitoneal injection of pentobarbital. After cannulation into the portal vein, the liver was perfused with calcium-free balanced NS containing 0.5 mg/mL collagenase and 1 mg/mL pronase E. Then the liver was removed, cut into small pieces and incubated in a solution containing 0.5 mg/mL collagenase. After washed, HSCs were purified by density gradient centrifugation with 18% Nycodenz, collected from the top layer, washed and suspended in DMEM supplemented with 10% FBS at the concentration of 1×10^6 cells/mL, and seeded on uncoated 24-well plastic plates at 1×10^5 /well supplemented with 20% FBS/DMEM for 24 h. Then the HSCs were subjected to tetrandrine treatment after cultivation in 2% FBS/DMEM for another 24 h. Over 90% of isolated HSCs were viable as assessed by trypan blue exclusion with a purity of higher than 90% as determined by direct cell counting under a phase-contrast microscope and intrinsic vitamin A autofluorescence.

HSCs were subjected to different treatments for 5 d as follows: control group: without any treatment; TGF β 1 group: supplemented with 5 ng/mL TGF β 1; M6P group: supplemented with 100 μ g/mL M6P and 5 ng/mL TGF β 1; RGD group: supplemented with 100 μ g/mL RGD and 5 ng/mL TGF β 1; RGD-M6P group: supplemented with 200 μ g/mL RGD-M6P and 5 ng/mL TGF β 1.

Immunocytochemical analysis of α -SMA

HSCs were cultured on 24-well plates with different treatments as described above. HSCs were fixed with ethanol/acetic acid after 5 d of treatment. α -SMA antibody, horse radish peroxidase-conjugated secondary

antibody and diaminobenzidine were added sequentially according to the standard protocol. Semi-quantitative assessment of protein expression was performed using pathological image analysis system. The expression of α -SMA was estimated by gray value.

PCIII content

PCIII in supernatant was measured with a radioimmunoassay kit according to the manufacturer's instructions.

Flow cytometry

The cell cycle was analyzed by flow cytometry to assess the proliferation rate of HSCs. Adherent and floating cells were collected from a 6-well plate, centrifuged and washed twice with PBS at room temperature. Cell pellets were re-suspended in ice-cold 70% ethanol for overnight fixation at 4 °C, centrifuged and re-suspended in 0.8 mL PBS. One microlitre 10 mg/ μ L RNase and 20 μ L propidium iodide (1 mg/mL) were added and samples were incubated at 37 °C for 30 min before analysis by flow cytometry using a Beckman Coulter FC500. Proliferation was quantitatively measured as the percentage of cells in the G₂/M phase.

Statistical analysis

All data were expressed as mean \pm SD. Statistical analysis of values was performed with SPSS software (10.0 version). $P < 0.05$ was considered statistically significant.

RESULTS

Morphological study of isolated HSCs *in vitro*

Over 90% of isolated HSCs were viable as assessed by trypan blue exclusion, which were small and round in appearance (Figure 1A). HSCs stimulated by 327 nm-length laser, showed a blue-green intrinsic autofluorescence due to vitamin-A rich droplets. After cultured for 72 h, HSCs presented a wall-adhesive growth pattern and many spindle-like or asteroid membranous processes and a reduction of vitamin-A droplets (Figure 1B). Overall, these findings indicated that HSCs freshly isolated from normal rats showed an activated form after 5 d of *in vitro* culture.

Expression of α -SMA in culture-activated HSCs

Immunocytochemical assay was used to evaluate the percentage of α -SMA-positive HSCs in different groups. The percentage of α -SMA-positive HSCs was significantly higher in TGF β 1 and M6P groups (Figures 2B and 2C)

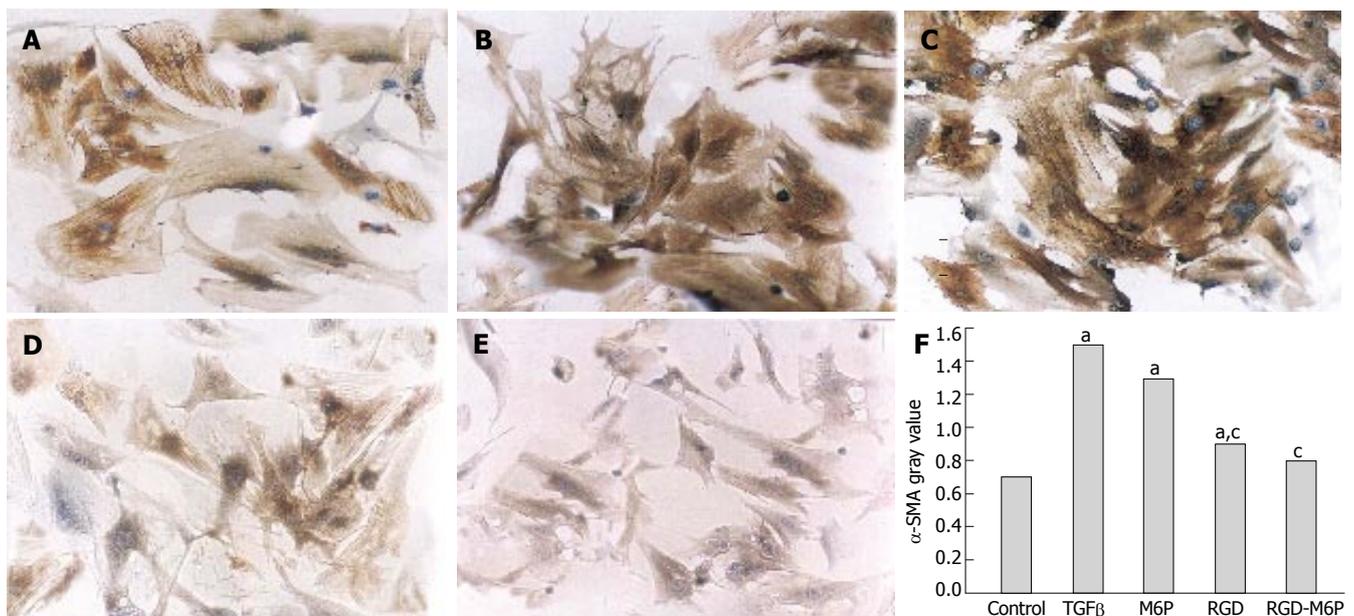


Figure 2 Immunocytochemical analysis of HSCs after cultured for 5 d (A), after treatment with TGF β 1 (B) and M6P (C), weak positivity of α -SMA RGD (D) and RGD-M6P (E), and elevated expression of α -SMA (F) in HSCs. ^a $P < 0.05$ vs control groups; ^c $P < 0.05$ vs TGF β 1.

Table 1 PCIII level in different groups (mean \pm SD)

| Group | n | Concentration of PCIII (ng/mL) |
|---------------------|---|-----------------------------------|
| Control group | 5 | 323.59 \pm 16.14 |
| TGF β 1 group | 5 | 403.56 \pm 7.97 ^a |
| M6P group | 5 | 399.70 \pm 2.25 ^a |
| RGD group | 5 | 370.87 \pm 13.07 ^{a,b} |
| RGD-M6P group | 5 | 357.61 \pm 13.07 ^{a,b} |

^a $P < 0.01$ vs control groups; ^b $P < 0.01$ vs TGF β 1 group.

than in control, RGD and RGD-M6P groups (Figures 2D and 2E). Even condensed α -SMA positive granules could be found (Figure 2B). No difference was found in the immunohistochemical study between RGD and RGD-M6P groups (Figure 2F). The cytomorphology study showed that the primary isolated HSCs were positive for α -SMA and the positivity was intensified through auto-activating characteristics of HSCs *in vitro*. Additionally, RGD and RGD-M6P could inhibit the activation of HSCs stimulated by TGF β 1.

PCIII excretion from culture-activated HSCs

PCIII is the most acceptable parameter of fibrogenesis as compared to other ECM contents, such as collagens I, III, IV, fibronectin, laminin and proteoglycans^[15,16]. The level of PCIII in the control group was lower than that in the other groups ($P < 0.01$, Table 1), and higher in TGF β 1 and M6P groups than in RGD and RGD-M6P groups. No difference was found in the level of supernatant PCIII between RGD and RGD-M6P groups. The results showed that the effect of RGD-M6P was better than that of M6P on inhibiting PCIII expression after stimulated by TGF β 1.

Proliferation of cultured HSCs

The activation of HSCs resulted in over-expression of

α -SMA and increased proliferation of HSCs. Cell cycle analysis was used to evaluate the proliferation rate by flow cytometry. The primary isolated HSCs showed a characteristic proliferation (Figure 3A), which was further increased by TGF β 1 (Figure 3B). M6P showed no effect on the proliferation of HSCs (Figure 3C). On the contrary, RGD and RGD-M6P inhibited the proliferation of isolated HSCs significantly (Figures 3D and 3E).

DISCUSSION

In the past decade, significant advances have been made in anti-fibrosis therapy due to a better understanding of the cellular and molecular mechanism of hepatic fibrogenesis^[1-3,8]. The key factors in the pathogenesis of liver fibrosis include the activation and proliferation of HSCs and their transformation into myofibroblasts^[5]. Therefore, HSCs are the important target for antifibrotic therapies^[4,12,17,18]. However, contrary to *in vitro* data, *in vivo* studies^[13,14,19] demonstrated that most antifibrogenic agents cannot be efficiently taken up by HSCs and the binding sites expressed on (activated) HSCs can serve as potential targets for carrier molecules. Two of these are integrin and M6P/IGF-II receptors and their expressions are increased on activated rat HSCs, particularly during fibrosis. Some anti-fibrotic reagents targeting at HSCs have been developed^[12,20], which allow cell-specific delivery of antifibrotic drugs to HSCs, thus enhancing their effectiveness *in vivo*.

Specific interactions between cells and ECM components are mediated by transmembrane proteins, especially heterodimeric integrins. Cell-matrix interactions guide or modulate cellular activities, such as adhesion, migration, differentiation, proliferation, and apoptosis^[13]. Most integrins can recognize and bind to the amino acid sequence of RGD present in various matrix proteins. The expression of integrins is increased during liver fibrosis,

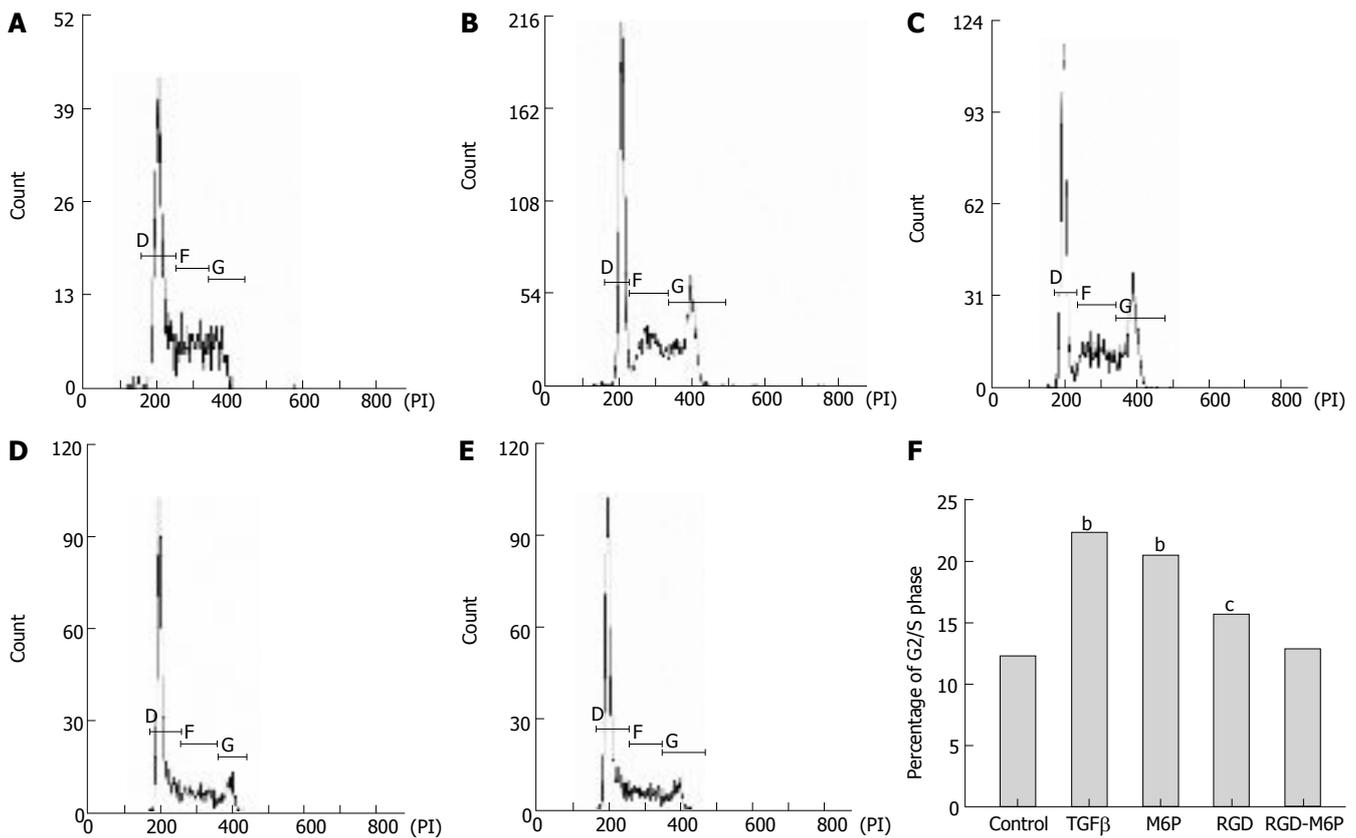


Figure 3 Flow cytometry analysis of cell cycle. PI histogram shows the details of cell cycle, in which the gates of D, F and G represent the G0/G1, S and G2/M phases, respectively, shown in corresponding figure. **A:** Proliferation of primary cultured HSCs; **B:** Increased proliferation of primary cultured HSCs in the presence of TGFβ1; **C:** Increased proliferation of primary cultured HSCs in the presence of M6P; **D** and **E:** Enlarged fraction of G2/M phase cells after treatment with RGD and RGD-M6P; **F:** Distribution of cell cycle phases was calculated using Multicycle I.O. ^b*P* < 0.01 vs control, RGD and RGD-M6P groups; ^c*P* < 0.05 vs control.

especially on the membrane of activated HSCs, suggesting that integrins can be used as a homing device to target at HSCs in fibrotic livers^[19,21]. About 10%-20% of M6P/IGF-II receptors are expressed in cell membrane and over-expressed with the activation of HSCs^[14]. It was reported that integrin antagonists can reduce the level of serum PCIII in fibrotic rats and inhibit the stimulating action of TGFβ1 on ECM expression^[22-24]. Beljaars *et al*^[25,26] showed that M6P-modified albumin accumulates in slices of normal and fibrotic liver and is taken up by HSCs. In the present study, we synthesized RGD-M6P and investigated its effects on the activation and proliferation of the primary HSCs *in vitro*.

The isolated primary HSCs in culture undergo auto-activation^[27]. During this process, the expression of α-SMA is raised^[28] and HSCs transform into fibroblast-like cells with increased proliferating activity^[27], which might lack microenvironment *in vitro*. HSCs supplemented with TGFβ1 put forth more processes on their cell membrane, and the supernatant PCIII level is increased^[29,30]. At the same time, the proliferation of HSCs is increased. Both RGD and RGD-M6P could inhibit the effect of TGFβ1 on the activation and proliferation of HSCs as well as PCIII expression. Though the M6P molecule participates in the activation of latent TGFβ1^[31], it can be used as a candidate in the study of anti-fibrosis. In the present study, we could not find its anti-fibrosis activity because M6P acted on the activated TGFβ1 rather than the latent form. Moreover,

RGD-M6P may have a more effective anti-fibrotic activity than RGD because RGD-M6P targets at the integrin and M6P/IGFII receptors on HSC membrane, thus more effectively modulating the HSC activity. But our present results failed to support the hypothesis. Further study is needed to explore the precise mechanism of this new complex.

In conclusion, integrins are related with TGFβ1 signal transduction and influence other signal transduction molecules such as JNK and NF-κB.

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RAPID COMMUNICATION

Detection of YMDD mutants using universal template real-time PCR

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Abstract

AIM: To establish a rapid and accurate method for the detection of lamivudine-resistant mutations in hepatitis B virus and monitor of lamivudine resistance during lamivudine treatment in patients with chronic hepatitis B virus infection.

METHODS: We established a real-time PCR method using a universal template and TaqMan probe to detect YMDD mutants. Variants of YVDD and YIDD were tested by individual reactions (reaction V and reaction I) and total hepatitis B viruses were detected in another reaction for control (reaction C). Results were determined by $\Delta Ct < 3.5$ ($\Delta Ct = Ct$ of reaction V or I - Ct of reaction C). Clones of the HBV polymerase gene containing different YMDD mutations were tested. Serum samples from 163 lamivudine-treated patients with chronic hepatitis B virus infection were detected using this method and the results were confirmed by DNA sequencing.

RESULTS: As many as 1000 copies per milliliter of wide-type plasmid were detected and nonspecific priming was excluded. In the 163 samples from patients treated with lamivudine, lamivudine-resistant mutations were detected in 51 samples.

CONCLUSION: This universal real-time PCR is a rapid and accurate method for quantification of YMDD mutants of HBV virus in lamivudine-treated patients and can be used to monitor lamivudine-resistant mutations before and during lamivudine therapy.

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Key words: HBV; YMDD; Mutation; UT-PCR

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INTRODUCTION

It is estimated that approximately 350 million persons worldwide are chronically infected with hepatitis B virus (HBV)^[1,2]. HBV is the leading cause of liver cirrhosis and hepatocellular carcinoma (HCC) and is responsible for 60 - 80% of all HCC cases^[2,3]. Treatment of HBV infection has been revolutionized by the introduction of antiviral drugs such as lamivudine. However, long-term monotherapy commonly does not result in complete suppression of viral replication and is associated with the emergence of resistant mutants^[4-7]. Specific mutations that result in replacement of methionine (M) in tyrosine-methionine-aspartate-aspartate (YMDD) motif of HBV reverse transcriptase (rt) by valine (V), isoleucine (I), or serine (S) confer resistance to lamivudine^[8].

Mutations leading to lamivudine resistance can be detected by direct sequencing of HBV DNA after PCR amplification of a selected part of the viral polymerase gene. However, this is expensive, laborious and time-consuming with low level of sensitivity, usually detecting 20% the total virus population^[9]. Other molecular techniques, such as restriction fragment length polymorphism (RFLP)^[10], 5' nuclease assay^[11], line probe assay^[12], peptide nucleic acid-mediated PCR clamping^[13,14], and oligonucleotide chips^[15], overcome some of the limitations of DNA sequencing, but they are also time-consuming and expensive. Recent studies using real-time PCR have obtained quantitative results but cannot avoid nonspecific amplification^[16,17].

In the present study, we established a rapid and accurate method for the detection of lamivudine-resistant mutations in HBV based on real-time PCR using a universal template. Positive and negative controls were included to evaluate the quality of the assay.

MATERIALS AND METHODS

Patients and extraction of HBV DNA

Serum samples were collected from 163 patients with chronic HBV infection who had received lamivudine monotherapy for one to two years. The ethical committee of our hospital approved the study and oral consent was obtained from the patients. HBV DNA was extracted from serum samples using the QIAamp blood kit (Qiagen,

Table 1 Primers and probe used in universal RT-PCR

| Primers or probe | Sequence |
|------------------|---|
| Reverse primers | |
| Reaction V | 5'-CCCCCAATACCACATCATCC-3' |
| Reaction I | 5'-CCCCCAATACCACATCATCA-3' |
| Reaction C | 5'-CCCCCAATACCACATCATC-3' |
| Forward primers | 5'-tgaggagcagagacggaagtATACAA CACCTGTATCCCATCCCAT-3' |
| Probe | 5'-FAM ACTTCCGTCCTCGTGCTCTCA TAMRA-3' |

Chatsworth, California) as described elsewhere^[11].

Primers and probe

The primers and probe used in this study are summarized in Table 1. Two different reverse primers that would selectively amplify the YVDD (rtM204V) and YIDD (rtM204I) quasispecies and a common reverse primer to a highly conserved sequence within the polymerase open reading frame were used to discriminate different mutants. The amplicon was detected by real-time PCR with a TaqMan probe that annealed to the universal template linked to the primers. A universal forward primer was used in the control for amplification of all kinds of quasispecies. A universal template was linked to each primer as described previously^[18]. In brief, the universal template (UT) sequence with a size of 21 bp was attached to the 5' end of the primers specific to YMDD variants^[17]. The UT probe was labeled with 6-carboxyfluorescein (FAM) at the 5' end and DABCYL quencher at the 3' end. During the annealing phase, the UT probe specifically annealed to the 5' end of the UT-PCR primer and the 3' end of the UT-PCR primer specifically annealed to the target sequence and was extended. Due to the 5' exonuclease activity of DNA polymerase, the hybridized UT probe was hydrolyzed, leading to the separation of the reporter moiety from the quencher moiety and the generation of a fluorescent signal. The signal could be detected by ABI 7000 real-time PCR system (Applied Biosystems Inc. California, USA).

Real-time PCR

The amplification was performed on ABI 7000 with a final volume of 50 μ l by incubating the reaction mixture at 50 °C for 2 min and at 95 °C for 5 min, followed by 40 cycles of PCR amplification at 94 °C for 20 s and at 55 °C for 30 s. The reaction mixture contained the following components: 1 \times PCR buffer, 100 nmol/L primers and probes, 400 μ mol/L each of dATP, dGTP, dCTP and dUTP, 1.5 units of hot-start Taq DNA polymerase, 0.2 units of Amperase uracil N-glycosylase (UNG), 3 mmol/L MgCl₂, 20 mmol/L KCl. The amplification was optimized in Fosun Diagnostics (Fosun Diagnostics, Shanghai, China). Variants of YVDD and YIDD and total HBV virus were amplified in individual reactions (reactions V, I and C for YVDD, YIDD and HBV virus respectively). The positive results were determined by Δ Ct < 3.5. Δ Ct = Ct of reaction V or I - Ct of reaction C.

DNA sequencing

The DNA sequence of the domain C of HBV polymerase

gene was analyzed as described elsewhere^[11]. In brief, HBV DNA extracted from serum samples was amplified by PCR. PCR products were purified and sequenced by ABI 310 sequencer (Applied Biosystems Inc., California, USA)

RESULTS

Specificity of primers and probe

Mismatched templates and primers (mutant primers to wild-type templates and vice versa) were used to validate this assay. Dilution series of 10⁸, 10⁶, 10⁴ copies per milliliter were used and PCR was performed with the mismatched primers. Nonspecific priming to the alternate template was observed at the template concentrations of 10⁶ and 10⁸ copies per milliliter but not at 10⁴ copies per milliliter. Similar to previous study^[17], the degree of cross-priming was at least 4 logs. For exclusion of nonspecific priming, the positive results were determined by Δ Ct < 3.5. Δ Ct = Ct of reaction V or I - Ct of reaction C.

Sensitivity and limit of detection

Mixing experiments were performed to determine the sensitivity of the assay. Mutant and wild-type plasmids were mixed with the ratio of 0:1, 1:1, 1:10, 1:100 and 1:1000 at end concentrations of 10⁵, 10⁶, 10⁷ and 10⁸ copies per milliliter respectively (five replicates for each mixture). In all the mixtures, the detection limit of the assay was 1000 copies per milliliter.

Comparison of real-time UT-PCR and sequencing

The assay was evaluated using serum from 163 lamivudine-treated patients with chronic HBV infection. All the patients treated with lamivudine alone for one to two years were HBV DNA positive by real-time PCR. Real-time UT-PCR was performed with all the samples together with positive and negative controls. Fifty-one (31%) of 163 samples were detected with YVDD (Figure 1) or YIDD mutants (YMDD in 112, YIDD in 21, YVDD in 27 and YIDD + YVDD in 3). YMDD mutations were detected in 19% (14 of 73) of the patients treated with lamivudine for one year and in 40% (37 of 90) of the patients treated for two years. The results of real-time UT-PCR and sequencing were concordant in 15 randomly selected samples (Table 2).

DISCUSSION

A more accurate and rapid method than DNA sequencing and RFLP is needed to detect and quantify the YMDD mutants in sera of chronic HBV infection patients before and during lamivudine therapy. A real-time UT-PCR assay has been developed for detection of YMDD mutations in a large number of patients. The detection limit of this assay is 1000 copies per milliliter of mutants and 1% within a mixed virus population at a concentration of 10⁵ copies per milliliter of serum. This real-time UT-PCR assay is more sensitive than DNA sequencing. In addition, the result of the assay can be easily determined by the difference between Ct values of the mutant and the control and no quantitative standards are needed.

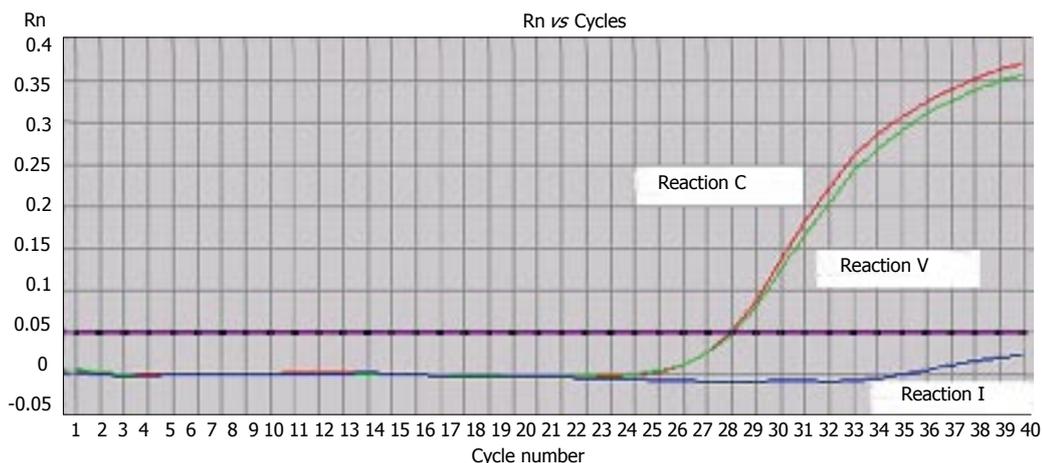


Figure 1 Detection of YMDD mutants using real-time UT-PCR. Three parallel reactions showed YVDD mutant.

Table 2 Comparison of results obtained by real-time PCR and sequencing

| Samples | HBV DNA (Copies/mL) | Result of UT-PCR | Result of sequencing |
|---------|------------------------|------------------|----------------------|
| 1 | 2.49 × 10 ⁵ | YMDD | 5' -TATATGGATGAT-3' |
| 2 | 4.73 × 10 ⁷ | YIDD | 5' -TATATTGATGAT-3' |
| 3 | 6.10 × 10 ³ | YMDD | 5' -TATATGGATGAT-3' |
| 4 | 6.24 × 10 ⁶ | YIDD | 5' -TATATTGATGAT-3' |
| 5 | 2.98 × 10 ⁹ | YIDD | 5' -TATATTGATGAT-3' |
| 6 | 8.13 × 10 ⁶ | YMDD | 5' -TATATGGATGAT-3' |
| 7 | 1.35 × 10 ⁴ | YMDD | 5' -TATATGGATGAT-3' |
| 8 | 3.57 × 10 ³ | YMDD | 5' -TATATGGATGAT-3' |
| 9 | 2.74 × 10 ⁶ | YVDD | 5' -TATGTGGATGAT-3' |
| 10 | 9.10 × 10 ⁴ | YVDD | 5' -TATGTGGATGAT-3' |
| 11 | 7.02 × 10 ⁴ | YMDD | 5' -TATATGGATGAT-3' |
| 12 | 5.18 × 10 ⁵ | YMDD | 5' -TATATGGATGAT-3' |
| 13 | 3.51 × 10 ³ | YMDD | 5' -TATATGGATGAT-3' |
| 14 | 8.04 × 10 ⁴ | YMDD | 5' -TATATGGATGAT-3' |
| 15 | 4.29 × 10 ⁶ | YMDD | 5' -TATATGGATGAT-3' |

Real-time UT-PCR has two major advantages, namely the universal probe can be used for many target sequences and it is less expensive compared with other sequence-specific fluorescent PCR techniques^[18]. In the present study, different YMDD variants were detected using different primers specific to the target DNA and a probe specific to the universal template, showing that it is cost-effective and convenient to detect different sequences simultaneously.

The overall prevalence of the YMDD mutations in the patients treated with lamivudine for one and two years in this study was 19% (14 of 73) and 40% (37 of 90), respectively, which is consistent with prevalence reported by previous studies^[4,5,19]. Mixture of YIDD and YVDD variants was observed in three samples.

In the present study, the primer used to detect rtM204I was specific to sequence ATT. Sequences of ATG and ATC could not be detected by this assay. However, this could be improved by adding two reverse primers specific to ATG and ATC into the reaction used to detect YIDD (data not shown).

Several real-time PCR methods are available for the detection of YMDD mutations^[16,17,20,21]. Selective primers have been used^[16,17]. One of the most important

advantages of this study is that the result of mutants could be easily determined by the difference between Ct values of the mutant and the control without additional quantitative standards. Furthermore, nonspecific priming could be excluded by the cut-off of $\Delta Ct < 3.5$ because the degree of cross-priming was at least 4 logs in this study and elsewhere^[17]. The impact of the intra-assay variability is reduced in this assay. However, this arbitrarily determined cut-off value reduces the sensitivity of the assay. The real-time UT-PCR is cost effective and convenient for large scale screening in clinical practices.

In conclusion, the real-time PCR assay established in this study is a rapid and accurate tool for detection of lamivudine-resistant mutations before and during lamivudine therapy.

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S- Editor Wang J L- Editor Wang XL E- Editor Bi L

RAPID COMMUNICATION

Functional evaluation of a new bioartificial liver system *in vivo* and *in vitro*

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liver system *in vitro* and *in vitro*. *World J Gastroenterol* 2006; 12(8): 1312-1316

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INTRODUCTION

Acute hepatic failure (ALF) is a disease with a high mortality. Although significant improvements have been achieved in critical care therapy, the mortality rate of ALF patients is about 80%. In many patients liver failure is reversible and if short-term liver support is provided, the liver may regenerate. Survivors may recover full liver function and a normal life expectancy. For many years, liver transplantation has been the only curative treatment for this condition, subjecting many patients to replacement of a potentially self-regenerating organ, with the lifetime danger of immunosuppression and its attendant complications, such as malignancy. Additionally, because of the shortage of livers available for transplantation, many patients die before a transplant can be performed, or are too ill to undergo surgery at the time a liver becomes available. The survival of patients excluded from liver transplantation or those with potentially reversible ALF might be improved with temporary artificial liver support. Among a variety of liver support therapies, bioartificial liver (BAL) therapy is marked as the most promising solution. Some BAL devices are under trials in animals^[1-4] and human beings^[5-8]. The major problem is how to configure a BAL system. To attain enhanced efficacy of liver support, several BAL configurations have been proposed. The long-term maintenance of hepatocyte function is crucial for any BAL system. Some systems use hepatocytes attached to microcarrier beads or multicellular spheroid aggregates^[9-11]. In regard to bioreactor design, various bioreactor configurations have been proposed that employ glass plates, hollow fiber membranes, encapsulation in biological matrices, and 3-dimensional carrier materials^[12-15].

In this study, porcine hepatocytes were isolated by *in situ* recirculating collagenase perfusion method and cultured with spinner culture method to prepare hepatocyte spheroids. A new BAL system was configured by inoculating hepatocyte spheroids into cell circuit of a BIOLIV A3A hollow fiber bioreactor and the functions of the BAL system were evaluated *in vitro* as previously described^[16,17]. At same time, the efficacy evaluation of the

Abstract

AIM: To evaluate the functions of a new bioartificial liver (BAL) system *in vitro* and *in vitro*.

MEHTODS: The BAL system was configured by inoculating porcine hepatocyte spheroids into the cell circuit of a hollow fiber bioreactor. In the experiments of BAL *in vitro*, the levels of alanine aminotransferase (ALT), total bilirubin (TB), and albumin (ALB) in the circulating hepatocyte suspension and RPMI-1640 medium were determined during 6 h of circulation in the BAL device. In the experiments of BAL *in vitro*, acute liver failure (ALF) model in canine was induced by an end-side portocaval shunt combined with common bile duct ligation and transaction. Blood ALT, TB and ammonia levels of ALF in canines were determined before and after BAL treatment.

RESULTS: During 6 h of circulation *in vitro*, there was no significant change of ALT, whereas the TB and ALB levels gradually increased with time both in the hepatocyte suspension and in RPMI-1640 medium. In the BAL treatment group, blood ALT, TB and ammonia levels of ALF in canines decreased significantly.

CONCLUSION: The new BAL system has the ability to perform liver functions and can be used to treat ALF.

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Key words: Bioartificial liver; Liver transplantation; Porcine hepatocyte

Chen Z, Ding YT. Functional evaluation of a new bioartificial

BAL system was conducted in a model of ALF canines induced by an end-side portocaval shunt combined with common bile duct ligation and transaction.

MATERIALS AND METHODS

Materials

Chinese experimental male and female miniature pigs ($n=13$) weighing 2.5-4.0 kg, were provided by Beijing Agricultural University. The pigs were allowed free access to water and fasted for 12 h before the experiment. Hybrid male and female dogs ($n=16$) weighing 10-15 kg, were provided by the Experimental Animal Center of Drum Tower Hospital. Collagenase IV, RPMI-1640 medium, hepatocyte growth factor, nerve growth factor, and N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) were from Gibco BRL Life Technologies, Grand Island, NY, USA. Insulin, glucagon, transferrin, linoleic acid, glutamine, bovine serum albumin, Na_2SeO_3 , $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, poly (2-hydroxyethyl methacrylate) (poly-HEMA), penicillin, and streptomycin were from Sigma-Aldrich Chemie GmbH, Steinheim, Germany. BIOLIV A3A hollow fiber cartridges with abnormal molecular weight cut-off of 70 Ka, a pore size of 200 nm, and a surface area of 1.06 m^2 , were provided by Cell Biotech Ltd, Hong Kong, China.

Hepatocyte isolation and culture

Porcine hepatocytes were isolated by *in situ* recirculating collagenase perfusion method^[18]. Hepatocytes were inoculated at a density of $5 \times 10^7/\text{mL}$ in serum-free RPMI-1640 medium supplemented with 200 $\mu\text{g}/\text{L}$ hydrocortisone, 1 mg/L hepatocyte growth factor, 10 $\mu\text{g}/\text{L}$ nerve growth insulin, 4 $\mu\text{g}/\text{L}$ glucagon, 6.25 mg/L transferrin, 10 mg/L linoleic acid, 2 mmol/L glutamine, 0.5 g/L bovine serum albumin, 3 nmol/L Na_2SeO_3 , 0.1 $\mu\text{mol}/\text{L}$ $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 50 pmol/L $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 15 mmol/L N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 200 mg/L cefoperazone sodium, 100 000 U/L penicillin, and 100 mg/L streptomycin^[19]. To inhibit cell attachment and induce the formation of cell spheroids in suspension, 250 mL bottles were coated with poly-HEMA. About 100 mL of hepatocyte suspension was placed in each bottle. The bottles were placed in an incubator (50 mL/L CO_2 , 37 °C) for 20 h and slowly rotated (12 revolutions/h) as previously described^[20,21].

Configuration of BAL system

The BAL system was configured by inoculating the hepatocyte spheroids into the cell circuit of a hollow fiber bioreactor (BIOLIV A3A, Cell Biotech Limited, HK, China). The total cell circuit volume was 250 mL. Hepatocyte spheroids in serum-free RPMI-1640 medium containing 1.0×10^{10} primary porcine hepatocytes were infused into the outer space of the hollow fibers and the medium was circulated at 20 mL/min with continuous O_2 input (2 L/min) (Figure 1). The bioreactor was kept at 37.5 °C in an incubator^[16].

Canine ALF model

Canine ALF model was induced by end-side portocaval

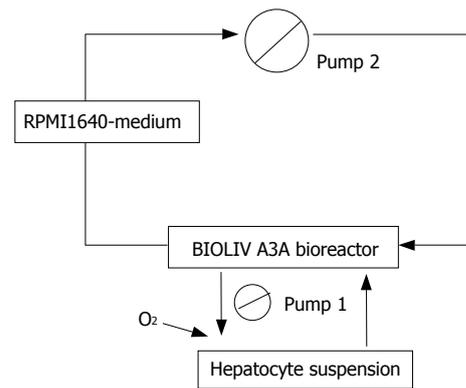


Figure 1 Schematic picture of the BAL system.

shunt combined with common bile duct ligation and transaction as previously described^[22]. All canines developed ALF 14 d after operation.

Determination of functions of BAL system *in vitro*

Independent experiments were performed 5 times with the BAL device. The RPMI-1640 medium flowed through the lumen of the hollow fibers at a rate of 30 mL/min. Samples of the hepatocyte suspension and RPMI-1640 medium were obtained at 2 h intervals during 6 h of circulation. Changes of alanine aminotransferase (ALT), total bilirubin (TB), and albumin (ALB) levels in the circulating hepatocytes and medium were determined with an automatic biochemical analyzer (Hitachi 7600, Japan).

Determination of functions of BAL system *in vivo*

Independent experiments were performed 8 times with the BAL device. Hemoperfusion was performed from femoral artery to femoral vein through the bioreactor at a rate of 30 mL/min. The ALF model canines were divided into two groups: the BAL treatment group ($n=8$) consisting of canine ALF models was perfused using the above serum-free medium inoculated with porcine hepatocyte spheroids for 6 h, and the control group ($n=8$) consisting of the canine ALF model was perfused using the above serum-free medium without porcine hepatocytes for 6 h.

Blood samples were obtained before (pre-circulation) and after the treatment (post-circulation). Blood ALT and TB were determined with an automatic biochemical analyzer (Hitachi 7600, Japan). Blood ammonia was determined with a biochemical analyzer (DT60II, Johnson and Johnson Medical Ltd., USA).

Statistical analysis

Results were expressed as mean \pm SD. Statistical differences were evaluated by analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

RESULTS

ALT, TB and ALB levels in hepatocyte suspensions and RPMI-1640 media during circulation

The average viability of the isolated hepatocytes was 97% by trypan blue exclusion. The formation of hepatocyte spheroids was observed after a culture of 20 h. In the hepatocyte suspensions, there were no significant changes of ALT during 6 h of circulation, whereas the TB and

Table 1 Changes of ALT, TBi and ALB in hepatocyte suspensions and RPMI-1640 media during circulation (mean±SD)

| Portion | Time (h) | Biochemical parameters | | |
|------------------------------|----------|------------------------|----------------------|-----------------------|
| | | ALT(U/L) | TB(μmol/L) | ALB(g/L) |
| Hepatocyte suspensions (n=5) | 0 | 13.7±3.5 | 3.5±2.4 | 2.8±1.5 |
| | 2 | 15.2±4.5 | 5.0±1.6 ^c | 3.1±1.6 |
| | 4 | 17.9±4.3 | 6.2±1.8 ^c | 3.6±0.9 ^c |
| | 6 | 18.4±3.2 | 8.4±2.6 ^c | 3.8±1.2 ^c |
| RPMI1640 media (n=5) | 0 | 0.8±0.2 ^a | 0.0±0.0 ^c | 0.5±0.1 ^a |
| | 2 | 1.5±0.5 ^a | 4.8±1.3 ^c | 1.8±0.3 ^{ac} |
| | 4 | 1.3±0.3 ^a | 5.7±1.6 ^c | 2.5±0.2 ^{ac} |
| | 6 | 1.4±1.0 ^a | 6.8±2.5 ^c | 2.8±0.3 ^{ac} |

^a*P*<0.05 vs hepatocyte suspensions at the same circulation time, ^c*P*<0.05 vs 0 h.

ALB levels increased gradually (*P*<0.05). The pre-circulation levels of ALT, TB and ALB were lower in the RPMI-1640 media than in hepatocyte suspensions (*P*<0.05). During 6 h of circulation, there was no significant change of ALT, whereas the TB and ALB levels gradually increased (*P*<0.05). At 2, 4, and 6 h of circulation, there were no significant differences of TB levels in RPMI-1640 media and hepatocyte suspensions, but the ALB concentration was lower in RPMI-1640 media than in hepatocyte suspensions at all times (*P*<0.05, Table 1).

Changes of biochemical parameters in ALF canines after BAL circulation

The two groups were comparable in terms of body weight (12.6±2.2 kg in BAL group and 13.2±2.1 kg in controls) and biochemical parameters before circulation. The parameters after circulation are shown in Table 2. In the BAL treatment group, blood ALT, TB and ammonia levels significantly decreased from 455.1±225.2 U/L, 74.6±25.4 μmol/L and 131.2±28.3 μmol/L before circulation to 273.3±151.3 U/L, 33.1±11.7 μmol/L and 33.4±21.7 μmol/L after circulation. While in the control group, there were no significant differences in blood ALT, TB and ammonia levels between pre-circulation and post-circulation, though these indices were declined slightly (*P*>0.05). Blood ALT, TB and ammonia levels were lower in BAL group than in control group after circulation (*P*<0.05). The viability of hepatocytes was about 90% at the end of BAL treatment.

Survival rate of ALF canines

The survival rates of ALF canines 7 d after treatment were 100% (8/8) and 62.5% (5/8) in BAL group and control group respectively with no significant difference (*P*>0.05).

Adverse reaction related to BAL treatment

The ALF canines had no apparent signs of toxicity during and after BAL treatment.

DISCUSSION

Treatment of ALF is a formidable clinical challenge. Currently, liver transplantation is the most effective therapy. Although advances have been achieved in

Table 2 Changes of biochemical parameters before and after circulation in two groups (mean±SD)

| Group | Biochemical parameters | | |
|---------------------|--------------------------|-------------------------|-------------------------|
| | ALT(U/L) | TB(μmol/L) | NH3(μmol/L) |
| BAL group (n=8) | | | |
| Pre-model | 18.2±12.6 | 2.8±1.2 | 1.0±0.0 |
| Pre-circulation | 325.8±54.7 ^c | 72.6±23.5 ^c | 132.2±28.3 ^c |
| Post-circulation | 212.3±42.3 ^{ac} | 33.4±22.2 ^{ac} | 33.2±21.7 ^{ac} |
| Control group (n=8) | | | |
| Pre-model | 16.6±38.3 | 2.6±1.9 | 1.0±0.0 |
| Pre-circulation | 300.2±52.6 ^c | 74.3±23.7 ^c | 126.3±30.4 ^c |
| Post-circulation | 280.1±45.9 ^c | 65.5±35.6 ^c | 100.3±13.5 ^c |

^a*P*<0.05 vs pre-circulation, ^c*P*<0.05 vs pre-model.

transplantation techniques, donor organ shortage remains a serious problem. The mortality of ALF is still high. However, many ALF patients can recover through liver regeneration after short-term liver support, which has prompted the design of an extracorporeal liver support device to “bridge” patients over until they either recover or receive a liver transplant. Among these devices, the BAL system is most promising. Various types of hepatocytes are inserted and different device designs have been proposed [25-28]. The BAL system differs from non-biologic artificial liver devices in the synthesis of essential metabolites and the selective removal of toxic substances, which are carried out by the cultured hepatocytes. An ideal BAL system can provide all the hepatic functions.

In this study, we configured a new BAL system by inoculating porcine hepatocyte spheroids into the cell circuit of a hollow fiber bioreactor and evaluated its functions *in vitro* and *in vivo*.

Nagaki *et al* [26] demonstrated that primary hepatocytes are superior to transformed hepatocytes as a source of biotransformation functions in the BAL system. The BAL system we developed uses viable porcine hepatocytes in the bioreactor to exert liver functions.

Since 10-30% of hepatocytes in the normal human liver are needed to maintain normal hepatic functions [27], Matsmura *et al* [28] proposed that a BAL requires 150-450 g of liver. Our BAL device contains 1.0×10¹⁰ hepatocytes to meet the need of patients with ALF. The liver of an adult canine weighs about 335 kg and contains 3.35×10⁹ hepatocytes. Therefore, the BAL device could be used in this experiment.

It has been reported that cell-cell interaction has an important role in maintaining the viability and the functions of hepatocytes [29]. In the present study, hepatocytes were incubated in serum-free medium and poly-HEMA-coated bottles by a continuous rotational method in order to restrict their attachment to the wall and promote the formation of hepatocyte spheroids. This method could not only facilitate cell-cell interaction and maintain cell functions, but it also meet the requirement of high-density culture in BA, thus reducing the possibility of immunoreaction by using serum-free medium with hormone and various growth factors [19].

The ideal bioreactor should provide a good environment for growth and metabolism of hepatocytes

as well as the efficient exchange of substances. At present, the most commonly used device is a hollow fiber bioreactor with many small hollow fibers made from semipermeable membranes. The device has two independent compartments that are separated by hollow fiber semipermeable membranes. The intratubular space is used to perfuse blood, while the extratubular space is used to culture hepatocytes. The blood or plasma of patients flows into the bioreactor, exchanging substances with hepatocytes through the semipermeable membranes. The membranes also provide immuno-isolation. In view of the molecular weight of albumin (68 ku), a hollow fiber membrane with 200 nm pore size and a MWCO of 70 ku was chosen for this study. The membrane allows passage of some relatively small molecules such as albumin, but restricts the passage of lymph cells and high molecular weight proteins. Blood-borne toxins and metabolic precursors are free to diffuse across the membrane to hepatocytes where they are metabolized. Metabolic products and detoxified toxins are free to diffuse back across the membrane to the flowing blood. Hepatocytes in the bioreactor also synthesize molecules (proteins, coagulation factors, enzymes, carrier molecules) that pass across the hollow fiber cartridge into blood. Continuous O₂ input could ensure hepatocytes to gain sufficient oxygen. Therefore, our BAL device has such a novelty as it uses hepatocyte spheroids and serum-free medium compared to previously reported BAL^[13-16].

Experiments of BAL *in vitro* showed that TB and ALB levels in hepatocyte suspensions and in RPMI-1640 media increased during 6 h of circulation. There were no significant differences of TB levels in RPMI-1640 medium and hepatocytes suspensions after 2 h of circulation. ALB level was lower in RPMI-1640 samples than in hepatocyte suspensions at all periods of circulation. ALB levels in RPMI-1640 medium and hepatocyte suspension averaged 2.8 g/L and 3.8 g/L, respectively, after 6 h of circulation. During the 6 h of circulation, there were no significant changes of ALT levels in RPMI-1640 medium or hepatocyte suspensions. ALT level was lower in RPMI-1640 medium than in hepatocyte suspensions at all time points. Bilirubin and albumin could readily cross the semi-permeable membrane because of their low molecular weights. The results indicate that albumin synthesized by hepatocytes in the BAL system can cross into the circulating stream in the intratubular space of hollow fibers.

In the experiments of BAL *in vivo*, blood ALT, TBi and ammonia levels significantly decreased after 6 h of circulation in the BAL group. There were no significant differences in blood ALT, TB and ammonia between pre-circulation and post-circulation in the control group, though these indices were slightly decreased after circulation. These results suggest that our BAL system has the potential not only to protect against hepatocyte destruction but also to dilute excess ALT in the systemic serum, which is consistent with the previous report^[30]. The survival rate was higher in BAL group than in control group, but there was no statistical significance, which may be related to the quantity of the samples. The viability of

hepatocytes was about 90% at the end of BAL treatment, indicating that our BAL device has its advantages.

In conclusion, the new BAL system configured in this research has a certain liver support effect and appears to have potential advantages for its clinical use in patients with ALF.

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Small bowel adenocarcinoma in Crohn's disease: A case report and review of literature

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Abstract

Small bowel adenocarcinomas are remarkable for their rarity, difficult diagnosis and poor prognosis. Here we report an unusual case of a 33-year-old patient in whom infiltrative adenocarcinoma of the small bowel was diagnosed after a 10-year history of Crohn's disease. In most previously reported cases, detection of Crohn's disease was subsequent to that of carcinoma of the small bowel or the patients involved had an even longer history of the disease. Our literature review suggests that the risk of small bowel adenocarcinoma is higher in patients with Crohn's disease than in the overall population. We present details on epidemiology as well as clinical and diagnostic aspects of this rare disease entity.

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Key words: Crohn's disease, Small bowel adenocarcinoma, Case report

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INTRODUCTION

The incidence of inflammatory bowel disease (IBD) is increasing since World War II with levels around 6/100 000 for Crohn's disease (CD) and 15 to 20/100 000 for ulcerative colitis, a marked rise in the age group between twenty to forty years for both entities^[1].

The ulcerations occur primarily in the small and large intestines, but may appear anywhere in the digestive tract from the mouth to the anus. Common symptoms of CD are abdominal pain, often in the lower right area, and

diarrhoea, but rectal bleeding, weight loss and fever may also appear. Children with CD may suffer stunted growth and delayed development. The severity of the symptoms fluctuates erratically over time. Patients experience flare-ups between intervals of remission or reduced symptoms. The causes of this disease have not been identified yet; but both genetic factors that induce continued abnormal activation of the immune system^[2,3] and environmental triggers, like *Mycobacterium avium* subspecies *paratuberculosis*^[4], are likely to be involved.

Oral or topical preparations of 5-aminosalicylates represent first line therapy, and steroids and azathioprine are used in severe cases; metronidazole and TNF-alpha antibodies are used in fistulating disease. Fifty to seventy percent of Crohn's patients undergo surgery for progression of disease indicated by the presence of fistulas, tumor in the abdomen and development of ileus.

IBD is linked to large and small bowel carcinoma, especially to adenocarcinomas^[5-7]. In the last twenty years, colorectal cancer has become the fourth most common cancer worldwide, and in Europe colorectal cancer represents the second most frequent cause of death from any cancer in men^[8]. Even though only about 1% of all colorectal cancers is associated with ulcerative colitis or Crohn's colitis, the risk of colorectal cancer for any ulcerative colitis patient is found to be 2% at 10, 8% at 20 and 18% at 30 years, duration of disease, regardless of disease extent^[9,10].

Small bowel carcinomas are uncommon representing only 1% to 5% of all gastrointestinal tract malignancies. The first observations suggested that particularly surgically bypassed bowel segments were exposed to high risk of small bowel adenocarcinoma^[11,12]. However, the risk of small bowel carcinoma in patients with CD is much higher, being up to 60-fold of that in the general population^[13,14].

Neither clear risk factors nor methods for early diagnosis have been established by the few studies on this distinctly uncommon complication within this rare disease. Here we report on a patient in whom infiltrative adenocarcinoma of the ileum was diagnosed after a 10-year history of CD and also discuss possible risk factors, symptoms, feasible diagnostic approaches and treatment options on the basis of published reports.

CASE REPORT

A 33-year-old man presented in 1992 with recurrent pyrexia and abdominal pain but no diarrhoea. Enteroclysis

was performed and a diagnosis of ileal CD was made. His family history was negative for this disease. For persistent abdominal pain under therapy with 5-aminosalicylates, he was put on corticosteroids (prednisone, 12.5 mg daily). In the following four years the patient experienced repeated episodes of abdominal pain without diarrhoea.

Two years later, an abdominal ultrasound performed for reassessment of the disease, showed a thick intestinal loop from the left to the right upper abdomen. Unfortunately no further diagnostic or therapeutic steps were undertaken at that time.

In 2002, the patient, complaining of increasing abdominal pain, underwent ileocolonoscopy, which yielded no suspicious macroscopic or histopathological findings. Blood tests showed mild anaemia, signs of malabsorption (low proteins, phosphorous and iron deficiency) but none for inflammation. The recommended enteroclysis of the small bowel was not performed. In order to reduce corticosteroids, therapy with azathioprine was initiated (2 mg/kg weight/ per day).

In June 2003 he presented again with abdominal pain, vomiting and distended abdomen. Enteroclysis showed a dilated intestinal loop of the ileum, a pseudotumor in the right abdomen and two stenotic areas, one of which was high-grade and located in the right upper abdomen (Figure 1). Prednisone was increased to 50 mg/day, in addition to metronidazole and ciprofloxacin. Three weeks later, with a deterioration of obstructive symptoms, the patient underwent surgery.

The surgical specimens consisted of a 45 cm and a 7 cm long resected segments. Macroscopic examination showed high-grade inflammatory alteration of the ileum along a 20 cm segment and two upstream high-grade stenoses. The external surface of the diseased segment appeared brownish discoloured with adhering connective tissue. The internal surface of the ileum showed discontinuous mucous membrane, streaks of ulcers and whitened swelling of the bowel wall. Some foci had a 'cobblestone' appearance. The 7 cm long segment had a stenotic 'sandglass' formation.

Extensive histopathological examination in the first stricture revealed a poorly differentiated adenocarcinoma infiltrating the serosa, incipient infiltration of the mesenteric fat and lymphangiosis carcinomatosa as well as a metastatic peritoneal range. Tumor had seeded seven out of eleven lymph nodes examined. The resected specimen also showed adenoma's dysplasia adjacent to the carcinoma. Extended antral metaplastic lesions within agglutinated villi up to one margin were found.

The second specimen revealed proper margins of resection. In the centre of the macroscopic stricture was another focus of the adenocarcinoma. In addition, moderately florid inflammatory infiltrates with acute erosions were detected, compatible with Crohn's disease.

Immunohistochemistry was negative for NSE and chromogranin, only a few tumor cells were slightly positive for synaptophysin. CEA level determined only after surgery was 54.2 ng/ml (normal <50 ng/ml).

The patient recovered well from surgery and then underwent chemotherapy according to Folfox IV-scheme with oxaliplatin, 5-fluorouracil and leucovorin in six cycles.



Figure 1 Enteroclysis: dilated intestinal loop of the ileum; altered Crohn's area (big arrow), one high grade and one low grade tumor stenosis in the right upper abdomen (two small arrows).

The patient tolerated the therapy quite well.

The computed tomography (CAT-scan) in October 2004 revealed up to two centimetres enlarged mesenteric and up to one centimetre enlarged retroperitoneal lymph nodes, without any further evidence for metastases. In November 2004 the patient presented with headache, vertigo and ambliopia and was diagnosed with meningeosis carcinomatosa. Intrathecal chemotherapy with methotrexate was started. He is now undergoing intrathecal chemotherapy with sustained-released cytarabine.

DISCUSSION

According to Parkin *et al* the age-standardized incidence of small intestine cancer (ICD-10 C17) ranges from 0.2 to 2.4 for males and from 0.2 to 1.8 for females worldwide^[15]. The 'Statistik Austria' National Registry has 1384 documented cases of small intestine cancer between 1983-2000^[16].

The association of CD with small bowel carcinoma is uncommon and to date only about 130 cases of small bowel carcinomas in patients with CD have been reported in the literature since the first description of this disease entity in 1956^[17]. Cases and studies published in the last few decades, however, bear out from a 12-fold to an over 60-fold increased risk of small bowel cancer in CD^[13, 14, 18]. This is in contrast to publications that still emphasize the popular position that CD is primarily associated with carcinoma of the colon. Adenocarcinoma is the most common forms of all small bowel malignancy and there appears to be an increased risk for developing ileal carcinoma in CD patients^[19-23].

Most of ileal carcinoma in CD are located in strictures^[11, 24, 25] and are often incidentally diagnosed postoperatively as in our case report. The occult carcinomas in strictures pose a challenge to diagnostic investigations using conventional modalities such as small bowel series and upper and lower gastrointestinal endoscopy. CT is now considered the imaging modality of choice^[26-28], and a fat density target sign in CT^[29] is also getting greater attention as reliable marker for diagnosing CD or even small bowel carcinoma. Abdominal MRI^[30], double-contrast enteroclysis^[31] and endoscopy^[32], especially video wireless capsule endoscopy^[33, 34], are promising new diagnostic tools.

Other interesting characteristics are adjacent metapla-

sia, adenoma and epithelial dysplasia^[35-38], which underline the importance of further research with respect to sequence-dysplasia in ileal adenocarcinomas in relation to Crohn's disease.

Risk factors for small intestine carcinoma in CD are chronic active course with stricture, fistulas and onset of disease before the age of 30 years^[25,39,40]. Further reported risk factors are: early onset, age between 30 and 50 years, male sex and smoking^[13,14,41-45]. Therapy of CD with corticosteroids, azathioprine and TNF-alpha antibodies are also considered as potential risk factors. It has been suggested in previous studies that azathioprine, administered mostly combined with steroids to patients with a long history of Crohn's disease, frequent recurrence or those allergic to 5-aminosalicylates has a carcinogenic potential^[14,46-49]. In the light of these reports, it is interesting to raise the question whether azathioprine therapy initiated in our patient after normal ileocolonoscopy 18 mo before diagnosis of carcinoma might have contributed to the acceleration of the malignant disease. In contrast to azathioprine, 5-aminosalicylates are considered preventive against the development of large and small bowel adenocarcinoma in inflammatory bowel disease^[13,23,50-53]. Mesalazine is now used for treating light to moderate Crohn's colitis and ileitis postoperatively to maintain remissions, but its potential to prevent malignancy needs to be evaluated. TNF-alpha antibodies, also mostly combined with immunosuppression, are used in patients with refractory, steroid-dependent and fistulating CD. There is a theoretical risk of increased rate of malignancies due to antagonism of TNF-alpha, but to date there is no clear proof of such an effect^[54-56].

Prognosis of small bowel adenocarcinoma is poor, and the mortality at 1 and 2 years ranges from 30-60% dependent on the stage of the cancer^[13,21,57-59].

Further prognostic factors are based on histologic findings such as positive surgical margins, poor differentiation, depth of tumor invasion, positive lymph nodes and extramural venous spread in small bowel adenocarcinoma^[60].

CONCLUSION

Small bowel adenocarcinoma in Crohn's disease is rare and preoperative diagnosis continues to present challenges. Long-term prognosis is poor - all the more it is important to be vigilant. Patients with increased risk are those with longstanding complicated CD presenting with a 'de novo' clinical picture of obstruction. Male patients, in particular smokers, are considered to be at increased risk. Since the diagnosis is difficult to make, attending physicians must exercise a high level of clinical suspicion for operative cure. The preventive potential of 5-ASA in adenocarcinoma of the colon suggests that this drug should be preferred to azathioprine in patients to maintain remission.

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Bile duct hamartomas (von Mayenburg complexes) mimicking liver metastases from bile duct cancer: MRC findings

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Abstract

We present a case of a 72-year-old man with a common bile duct cancer, who was initially believed to have multiple liver metastases based on computed tomography findings, and in whom magnetic resonance cholangiography (MRC) revealed a diagnosis of bile duct hamartomas. At exploration for pancreaticoduodenectomy, liver palpation revealed disseminated nodules at the surface of the liver. These nodules showed gray-white nodular lesions of about 0.5 cm in diameter scattered on the surface of both liver lobes, which were looked like multiple liver metastases from bile duct cancer. Frozen section of the liver biopsy disclosed multiple bile ducts with slightly dilated lumens embedded in the collagenous stroma characteristics of multiple bile duct hamartomas (BDHs). Only two reports have described the MRC features of bile duct hamartomas. Of all imaging procedures, MRC provides the most relevant features for the imaging diagnosis of bile duct hamartomas.

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Key words: Bile duct hamartoma; Magnetic resonance cholangiography; Multiple liver metastases

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INTRODUCTION

Multiple bile duct hamartomas (BDHs) are small benign neoplasms of the liver, pathologically containing cystic dilated bile ducts embedded in a fibrous stroma^[1]. In almost all cases, these patients are believed to have multiple liver metastases following initial imaging^[2], and diagnosis of BDHs is frequently not considered until after liver biopsy^[3]. It is difficult to be certain that all of the lesions identified radiologically, such as ultrasonography (US), computed tomography (CT), are definitely BDHs, necessitating referral for open biopsy to exclude liver metastases. Although the magnetic resonance imaging (MRI) appearance has been reported in 14 cases previously, only two reports have been published for BDHs with illustration of magnetic resonance cholangiography (MRC) features^[3,4]. In this report, MRC may be useful to allow accurate characterization of the liver lesions, thereby permitting the diagnosis of von Mayenburg complexes to be made with careful analysis of the MRI, and MRC images.

CASE REPORT

A 72-year-old man was admitted to our hospital in September 2003 with a history of epigastralgia. The physical examination was unremarkable. His serum levels of hepatobiliary enzymes were increased, however, that of total bilirubin (T Bil, 0.7 $\mu\text{mol/L}$) and direct bilirubin (D Bil, 0.3 $\mu\text{mol/L}$) were normal. Tumor markers, carbohydrate antigen19-9 (CA19-9) and carcinoembryonic antigen (CEA) were almost within normal range. Ultrasonography showed a dilatation of the common bile duct (CBD) and intrahepatic bile duct. Endoscopic retrograde cholangiography (ERC) showed the irregular stenosis of the lower CBD (1.5 cm in length) and upper part of CBD was dilated. Biopsied specimens from this tumor histologically revealed adenocarcinomas. On the basis of these findings, we diagnosed this tumor as a carcinoma of the CBD. Additionally, multiple, very small and hypodense nodules with ring-like enhancement in both hepatic lobes were found on early phase of enhanced CT (Figure 1).

The lesions were too small to characterize accurately by either modality, but were consistent with disseminated liver metastases. On MRC images (TR 16 000 ms TE 107.8 ms), multiple irregularly delineated hyperintense nodules were seen, not communicating with the biliary tree (Figure 2). These findings suggested the bile duct hamartoma (BDH) (von Mayenburg complexes).

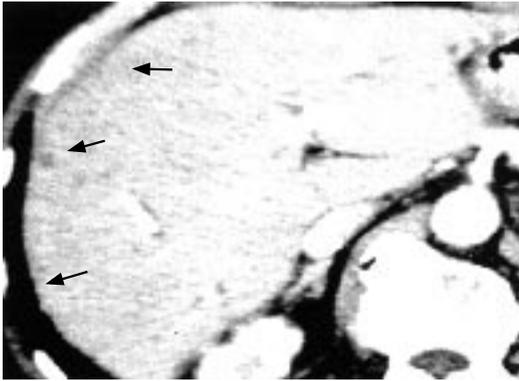


Figure 1 Contrast-enhanced computed tomography showing multiple, very small and hypodense nodules with ring-like enhancement (0.5 cm in diameter) in both hepatic lobes.

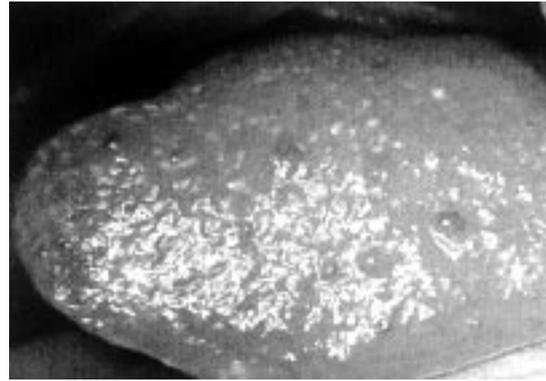


Figure 3 Intraoperative macroscopic view showing gray-white nodular lesions (about 0.5 cm in diameter) scattered on the surface of the both liver lobes.



Figure 2 MR cholangiography (TR 16 000 ms TE 107.8 ms) showing multiple irregularly delineated hyperintense nodules, not communicating with the biliary tree.

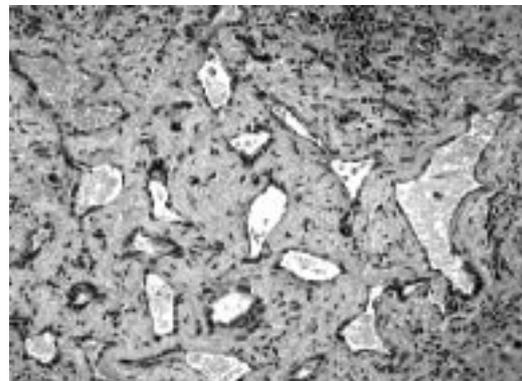


Figure 4 Microscopic view of liver biopsy showing multiple bile ducts with slightly dilated lumens embedded in the collagenous stroma (Hematoxylin and eosin, x100).

At exploration for pancreatoduodenectomy, liver palpation revealed disseminated nodules at the surface of the liver. These nodules showed gray-white nodular lesions (about 0.5 cm in diameter) scattered on the surface of the both liver lobes, which were looked like multiple liver metastases from the bile duct cancer (Figure 3). Frozen section of the liver biopsy disclosed multiple bile ducts with slightly dilated lumens embedded in the collagenous stroma characteristics of BDHs with no evidence of malignancy (Figure 4).

DISCUSSION

On gross pathology, Von Meyenburg complexes of the liver present as gray-white nodular lesions scattered throughout the liver parenchyma, usually measuring between 0.1 and 1.0 cm in diameter^[3]. In this case, enhanced CT showed low density small nodules with ring-like enhancement, therefore multiple liver metastases were suspected. The major differential diagnoses, especially on frozen section, are intrahepatic cholangiocarcinoma and metastatic adenocarcinoma. The clinical importance of bile duct hamartomas is its ability to mimic metastatic disease of the liver. Chamlou *et al*^[5] reported that, in the pancreatic cancer patient, the biliary duct hamartoma was erroneously confused intraoperatively with hepatic metastasis on frozen sections, and the pancreatoduodenectomy was therefore not performed at the first operation. Because of prevalence of biliary hamartomas, these entities should

be considered as possibilities when liver lesions are noted intraoperatively.

MRI can aid in the differentiation of liver cysts and liver metastases by identifying the hyperintense signal from liver cysts on heavily T2-weighted sequences. To our best of knowledge, only two reports have described the MRC features of BDHs^[3,4]. MRC can be extremely important in evaluating anomalies of bile ducts. Additionally, it allows differentiation between dilated biliary ducts, saccular dilatation of biliary duct system (Caroli's diseases), and periductal non-communicating cystic lesion, including polycystic disease, bile duct hamartomas and multiple abscess. In previous reports, MRC images showed multiple hyperintense cystic lesions of small diameter scattered in both liver lobes with normal appearance of the intrahepatic and extrahepatic bile ducts. No communication between the hamartomas and the intrahepatic bile ducts was observed. In this case, MRC findings are similar to the previous reports and compatible with BDHs. MRC offers the ability to characterize small liver lesions characteristics of BDH.

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Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

Genotypes: *gyrA*, *arg 1*, *c myc*, *c fos*, etc.

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| 1 | 4 days | 4 d | In figures, tables and numerical narration |
| 2 | 4 days | four days | In text narration |
| 3 | day | d | After Arabic numerals |
| 4 | Four d | Four days | At the beginning of a sentence |
| 5 | 2 hours | 2 h | After Arabic numerals |
| 6 | 2 hs | 2 h | After Arabic numerals |
| 7 | hr, hrs, | h | After Arabic numerals |
| 8 | 10 seconds | 10 s | After Arabic numerals |
| 9 | 10 year | 10 years | In text narration |
| 10 | Ten yr | Ten years | At the beginning of a sentence |
| 11 | 0,1,2 years | 0,1,2 yr | In figures and tables |
| 12 | 0,1,2 year | 0,1,2 yr | In figures and tables |
| 13 | 4 weeks | 4 wk | |
| 14 | Four wk | Four weeks | At the beginning of a sentence |
| 15 | 2 months | 2 mo | In figures and tables |
| 16 | Two mo | Two months | At the beginning of a sentence |
| 17 | 10 minutes | 10 min | |
| 18 | Ten min | Ten minutes | At the beginning of a sentence |
| 19 | 50% (V/V) | 500 mL/L | |
| 20 | 50% (m/V) | 500 g/L | |
| 21 | 1 M | 1 mol/L | |
| 22 | 10 μM | 10 μmol/L | |
| 23 | 1N HCl | 1 mol/L HCl | |
| 24 | 1N H ₂ SO ₄ | 0.5 mol/L H ₂ SO ₄ | |
| 25 | 4rd edition | 4 th edition | |
| 26 | 15 year experience | 15- year experience | |
| 27 | 18.5 kDa | 18.5 ku, 18 500u or M:18 500 | |
| 28 | 25 g.kg ⁻¹ /d ⁻¹ | 25 g/(kg·d) or 25 g/kg per day | |
| 29 | 6900 | 6 900 | |
| 30 | 1000 rpm | 1 000 r/min | |
| 31 | sec | s | After Arabic numerals |
| 32 | 1 pg L ⁻¹ | 1 pg/L | |
| 33 | 10 kilograms | 10 kg | |
| 34 | 13 000 rpm | 13 000 g | High speed; g should be in italic and suitable conversion. |
| 35 | 1000 g | 1 000 r/min | Low speed. g cannot be used. |
| 36 | Gene bank | GenBank | International classified genetic materials collection bank |
| 37 | Ten L | Ten liters | At the beginning of a sentence |
| 38 | Ten mL | Ten milliliters | At the beginning of a sentence |
| 39 | umol | μmol | |
| 40 | 30 sec | 30 s | |
| 41 | 1 g/dl | 10 g/L | 10-fold conversion |
| 42 | OD ₂₆₀ | A ₂₆₀ | "OD" has been abandoned. |
| 43 | One g/L | One microgram per liter | At the beginning of a sentence |
| 44 | A ₂₆₀ nm ^b P<0.05 | A ₂₆₀ nm ^a P<0.05 | A should be in italic. In Table, no note is needed if there is no significance instatistics: ^a P<0.05, ^b P<0.01 (no note if P>0.05). If ther is a second set of P value in the same table, ^c P<0.05 and ^d P<0.01 are used for a third set: ^a P<0.05, ⁱ P<0.01. |
| 45 | [*] F=9.87, [§] F=25.9, [#] F=67.4 | ¹ F=9.87, ² F=25.9, ³ F=67.4 | Notices in or under a table |
| 46 | KM | km | kilometer |
| 47 | CM | cm | centimeter |
| 48 | MM | mm | millimeter |
| 49 | Kg, KG | kg | kilogram |
| 50 | Gm, gr | g | gram |
| 51 | nt | N | newton |
| 52 | l | L | liter |
| 53 | db | dB | decibel |
| 54 | rpm | r/min | rotation per minute |
| 55 | bq | Bq | becquerel, a unit symbol |
| 56 | amp | A | ampere |
| 57 | coul | C | coulomb |
| 58 | HZ | Hz | |
| 59 | w | W | watt |
| 60 | KPa | kPa | kilo-pascal |
| 61 | p | Pa | pascal |
| 62 | ev | EV | volt (electronic unit) |
| 63 | Jonle | J | joule |
| 64 | J/mmol | kJ/mol | kilojoule per mole |
| 65 | 10×10×10cm ³ | 10 cm×10 cm×10 cm | |
| 66 | N·km | KN·m | moment |
| 67 | x±s | mean±SD | In figures, tables or text narration |
| 68 | Mean±SEM | mean±SE | In figures, tables or text narration |
| 69 | im | im | intramuscular injection |
| 70 | iv | iv | intravenous injection |
| 71 | Wang et al | Wang <i>et al.</i> | |
| 72 | EcoRI | EcoRI | <i>Eco</i> in italic and RI in positive. Restriction endonuclease has its prescript form of writing. |
| 73 | Ecoli | <i>E.coli</i> | Bacteria and other biologic terms have their specific expression. |
| 74 | Hp | <i>H pylori</i> | |
| 75 | Iga | Iga | writing form of genes |
| 76 | igA | IgA | writing form of proteins |
| 77 | ~70 kDa | ~70 ku | |

Sensitivity and inter-observer variability for capsule endoscopy image analysis in a cohort of novice readers

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Abstract

AIM: To determine the performance of novice readers (4th year medical students) for detecting capsule endoscopy findings.

METHODS: Ten capsule endoscopy cases of small bowel lesions were administered to the readers. Gold standard findings were pre-defined by gastroenterologists. Ten gold standard "targets" were identified among the 10 cases. Readers were given a 30-min overview of Rapid Reader software and instructed to mark any potential areas of abnormalities. A software program was developed using SAS to analyze the thumbnailed findings.

RESULTS: The overall sensitivity for detecting the gold standard findings was 80%. As a group, at least 5 out of 10 readers detected each gold standard finding per recording. All the gold standard targets were identified when the readers' results were combined. Incidental finding/false positive rate ranged between 8.2-59.8 per reader.

CONCLUSION: A panel of medical students with minimal endoscopic experience can achieve high

sensitivity in detecting lesions on capsule endoscopy. A group of novice readers can pre-screen recordings to thumbnail potential areas of small bowel lesions for further review. These thumbnails must be reviewed to determine the clinical relevance. Further studies are ongoing to assess other cohorts.

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Key words: Capsule endoscopy analysis; Small bowel lesions; Novice readers; Sensitivity

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INTRODUCTION

Capsule endoscopy is a new diagnostic procedure developed for the complete examination of the small intestine through video images transmitted from an ingestible camera^[1-3]. Briefly, the PillCamTM capsule endoscopy and diagnostic imaging system (GIVEN Imaging, Yoqneam, Israel) is a commercially available system consisting of three major components: PillCamTM capsule which captures images and transmits digital pictures (at 2 frames/s) over an 8-h period, sensor array and data recorder, which receives and records the data transmitted from the PillCamTM capsule and RAPIDTM Workstation, which is used to initialize the data recorder and to download and process the raw data from the data recorder^[4,5]. The processed information, composed of approximately 50 000 still images collected over an 8-h period, can be reviewed as a continuous video stream. The reported time range typically needed for a complete review of a single capsule endoscopy recording case is anywhere from 50^[6,7] to 120 min^[8].

Numerous studies have now demonstrated that the sensitivity and specificity of capsule endoscopy are advantageous over the traditional diagnostic methods of small bowel lesions^[5-7,9-11]. Capsule endoscopy may also reduce total medical utilization and costs as well as improve patient's quality of life in certain circumstances^[12].

One feature that can affect the diagnostic yield of capsule endoscopy is the image analysis process, i.e. the ability of the person reviewing the images (reader) to accurately detect significant lesions and interpret the findings. This process is time consuming and requires individuals to focus their undivided attention viewing the large number of images.

Currently, the process of capsule endoscopy image analysis has not been standardized with respect to the selection and training of individual readers, determination of the gold standard to which findings are compared to assess sensitivity and false positive rates or reporting of findings and diagnoses. Unfortunately, these important issues have not been well studied previously. Studies of inter-observer variability have been limited to anecdotal reports between 1 and 4 different readers^[13-20,23,24]. Furthermore, since capsule endoscopy image analysis is a time consuming process, the arduous process of image recognition and analysis is often delegated to individuals having received minimal pre-training with little consideration of their ability to achieve competency in reading capsule endoscopy recordings. A survey was conducted at the 2003 Given International Capsule Endoscopy Conference and found that 82% of gastroenterologists reported that they are the first readers to interpret the capsule endoscopy recordings, while 18% use a resident physician assistant and/or nurse to interpret the capsule endoscopy recordings first^[21].

In this clinical study, our aim was to determine the sensitivity, incidental finding and/or false positive rate, and intraclass correlation of novice capsule endoscopy readers who were 4th year US medical students with minimal endoscopic background for detecting pre-specified capsule endoscopy findings. Previous studies have shown that it is not a simple task to achieve 100% sensitivity on capsule endoscopy recording^[13-20]. In addition, it was reported that since the pathology is visualized in more than a small percentage of images from each capsule endoscopy recording, a fatigue gastroenterologist may analyze the capsule endoscopy recording at a very rapid speed, being likely to miss lesions^[14]. Hence, we propose that analysis of the same capsule endoscopy recording by multiple readers might be an effective method to achieve 100% sensitivity and decrease medical errors. If the combined results of the novice readers show a high sensitivity, then perhaps novice readers can be considered as physician extenders in analyzing capsule endoscopy recordings. In our study, instead of manually analyzing the capsule endoscopy readers' results, we used statistical software to perform the analysis in an attempt to decrease the time required for this process. The reason is that manual comparison of the readers' findings can be labor intensive and time consuming, if a large number of readers are evaluated. Finally, the method of using medical students as novice readers to analyze the images of a diagnostic modality has been described in the literature^[25].

MATERIALS AND METHODS

Capsule endoscopy recordings

Ten recordings with definitive sites of small bowel lesions were administered to the readers in a pre-set order

(lesions - AVM-3, small bowel tumor - 1, radiation enteritis - 1, ulcers/aphthous lesions - 3, and foreign body with ulceration - 2). Two gastroenterologists (attending physicians at the tertiary medical center) selected these 10 recordings.

Capsule endoscopy readers

The novice capsule endoscopy readers consisted of a group of ten 4th year medical students with minimal endoscopic background from David Geffen School of Medicine at UCLA (Los Angeles, CA, USA). All the participating medical students signed an informed consent agreement as approved by the local institutional review board.

The readers were blinded to the patients' clinical history because this study was to assess the readers' abilities to detect small bowel lesions on capsule endoscopy recordings rather than to test their medical knowledge. Furthermore, the readers were blinded to each other's capsule endoscopy findings.

Gold standard

The gold standard for the true positive findings was pre-defined by the two gastroenterologists (over 150 capsule endoscopy cases each at the time when this study was started) who independently reviewed all the available data for each of the 10 recordings, including pertinent medical history; previous endoscopic, radiologic and surgical examinations; the complete 8-h capsule endoscopy recording. The experts' consensus of positive findings was used to calculate the sensitivity and false positive measures for each individual reader as defined below. Ten gold standard "targets" were identified among the 10 cases. We did not include any negative findings in this study because our aim was to evaluate the readers' ability to detect positive findings. However, the readers did not know that there was at least one gold standard finding per case.

Capsule endoscopy image analysis

Each reader reviewed the entire 8-h recordings for all 10 cases to localize the thumbnailed significant lesions within the small intestine. Significant upper and lower gastrointestinal lesions could be detected by capsule endoscopy, but the lesions of esophagus, stomach, and colon were not analyzed. Presumably, lesions in these areas were detected during routine endoscopic evaluation.

The readers analyzed the 10 recordings in a consecutive order over a 30-90 d period. They were also asked to record how long it took for them to interpret each case and were told to use a cautious and highly inclusive approach, while interpreting the capsule endoscopy recordings in order to minimize the chance of missing any clinically significant lesions. All findings identified by each reader were marked, thumbnailed and annotated using the Rapid Reader software program (GIVEN Imaging, Yoqneam, Israel). The readers were given a 30-min overview of the Rapid Reader software and instructions for thumbnailing. Active gastrointestinal bleeding was often detected by the Suspected Blood Indicator program (two capsule endoscopy cases we used had active gastrointestinal bleeding lesions); however, we did not allow the readers to

Table 1 Gold standard findings and time intervals

| Location | Case | Time intervals | Findings |
|----------|------|----------------|--|
| 1 | 1 | 22 500–32 900 | Aphthous ulceration (from NSAIDS use) |
| 2 | 2 | 4 542–6 842 | Aphthous ulceration (from NSAIDS use) |
| 3 | 3 | 16 144–16 157 | Duodenal bleeding (AVM) |
| 4 | 4 | 25 302–25 372 | Staples and ulcerations (from prior Surgery) |
| 5 | 5 | 24 244–24 700 | Small bowel tumor |
| 6 | 6 | 23 596–23 692 | Aphthous ulceration (from Crohn's disease) |
| 7 | 7 | 32 657–50 000 | Radiation enteritis |
| 8 | 8 | 20 000–26 000 | Chicken bone |
| 9 | 9 | 1350–2434 | Bleeding angiodysplasia |
| 10 | 10 | 12 600–12 800 | Bleeding angiodysplasia |

use the Suspected Blood Indicator program on the Rapid Reader, since we wanted to assess the readers' true abilities to detect the lesions on capsule endoscopy recordings. Furthermore, we felt that active bleeding lesions on the capsule endoscopy recordings should be easily and consistently detectable by the readers.

Outcome accuracy measures and analysis

The percentage of cases where a reader had at least one finding in the gold standard areas of a case was expressed as the reader's sensitivity. If a reader had a finding outside the gold standard time interval, it was considered an incidental finding/false positive rate.

The time series for each case was divided into time intervals using the PROC MIXED procedure in SAS (SAS Institute Inc., Cary, NC, USA). Within each time interval, it was noted whether or not each reader had at least one finding. For a given time interval size, a time interval could be designated as being in a true problem area (part of the time interval was in the "gold standard" area for that case) or not. Each time interval could also be designated as having a "finding" or not, where the "finding" was yes if $X/10$ readers had a finding in that time interval. X was the reader's threshold. Reader's threshold was defined as the minimum number of readers out of all the readers who had to have a finding in a given time interval in order to consider it as a true positive finding (namely a true problem area). The clinical implication of the optimal time interval size and reader's threshold analyses was that this method could inform the capsule endoscopy readers, where the time series and the thumbnailed findings occurred the most. Therefore, the readers would know to which parts of the capsule endoscopy recording they needed to pay extra attention during the image analysis and review process. This is especially important and perhaps shortens the time needed for the gastroenterologists reviewing the thumbnailed findings of the screeners (in this case, the novice readers).

There were a total of 128 time interval size/reader's threshold combinations per case. In each of these time interval size/reader's threshold combinations, sensitivity and specificity were estimated for each case. Sensitivity was estimated as the probability of a finding, given the finding being in a region with a true finding (in the "gold standard" region), while specificity was estimated as the prob-

ability of no finding, given that the finding was not in an incidental/false positive region. Among the time interval size/reader's threshold combinations with 100% sensitivity, we calculated the incidental finding/false positive rate (the number of time intervals with a finding outside the gold standard area divided by the total number of time intervals outside the gold standard area) and the standardized number of minutes viewing incidental finding/false positive time intervals (the incidental finding/false positive rate multiplied by the average number of true negative time intervals for that reader's threshold/time interval size, multiplied by the time interval size in minutes). For each of these quantities, we calculated the average and the maximum value across all 10 capsule endoscopy cases. Separate analyses were conducted using different time interval sizes, which ranged in length from 20 to 25 000 s, in order to determine the impact of time interval size on the results.

Intraclass correlation was assessed separately for each case. The intraclass correlation among all 10 readers measured the agreement among the readers in their evaluation of capsule endoscopy recordings, above the agreement was expected by chance. The intraclass correlation coefficient was estimated for each time interval size and for each capsule endoscopy recording.

RESULTS

Based on the gold standard findings, 10 targets were specified in the 10 recordings used in this study (Table 1). The average time taken by the readers to interpret each case was 118 min. The overall sensitivity among the 10 readers was 0.80 (80%) for time interval size of 20 s. All findings were detected in 6 out of 10 readers. On a case level, the gold standard finding was identified by all the 10 readers in case #2 but only 5 readers for case #6. The individual reader sensitivity ranged between 60–100%, with reader #8 achieving 100% sensitivity, while reader #5 achieving only 60% sensitivity (Table 2). The readers were able to identify all the gastric, duodenal, and cecal images accurately. The number of incidental false positive finding ranged from a minimum of 8.2 in reader #1 to a maximum of 59.8 in reader #10 per recording (Table 3). By case, the number of incidental false positive findings ranged from 12 in case #9 to 40.1 in case #5. Intraclass correlation varied with case, but seemed to increase with increased time interval size. The overall intraclass correlation was <0.40 but cases #2 and #9, being low compared to fair agreement (Figure 1).

In the time interval size and reader's threshold analyses, the minimum time interval size for which sensitivity in all 10 recordings achieved 100% was 3 000 s. All possible reader thresholds achieved 100% sensitivity for all 10 recordings in at least one of the time interval sizes examined. The average percentage of incidental false positive findings in the 10 cases ranged from 28% with a reader's threshold of 7 and time interval of 5 000 s to 66% with a reader's threshold of 3 and a time interval of 10 000 s (Figure 2A). The maximum percentage of the incidental false positive findings in the 10 recordings ranged from 56% for time interval of 5 000 s and reader's threshold of 7–100% for several combinations (Figure 2B).

Overall, in the optimal time interval size and reader's threshold

Table 2 Findings and sensitivity

| Reader number | Case 1 | Case 2 | Case 3 | Case 4 | Case 5 | Case 6 | Case 7 | Case 8 | Case 9 | Case 10 | Total findings | Sensitivity |
|---------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|---------|----------------|-------------|
| 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 8 | 0.8 |
| 2 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 8 | 0.8 |
| 3 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 8 | 0.8 |
| 4 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 8 | 0.8 |
| 5 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 6 | 0.6 |
| 6 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 7 | 0.7 |
| 7 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 9 | 0.9 |
| 8 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 10 | 1 |
| 9 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 8 | 0.8 |
| 10 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 8 | 0.8 |
| Overall | 9 | 10 | 8 | 7 | 6 | 5 | 8 | 9 | 9 | 9 | 80 | 0.8 |

Average sensitivity = 80%.

Table 3 False positive/incidental findings

| Reader number | Case 1 | Case 2 | Case 3 | Case 4 | Case 5 | Case 6 | Case 7 | Case 8 | Case 9 | Case 10 | Min | Mean | Max | SD |
|---------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|---------|-----|------|-----|--------|
| 1 | 16 | 14 | 3 | 14 | 10 | 4 | 5 | 5 | 6 | 5 | 3 | 8.2 | 16 | 4.849 |
| 2 | 17 | 39 | 6 | 7 | 64 | 6 | 15 | 6 | 19 | 10 | 6 | 18.9 | 64 | 18.788 |
| 3 | 34 | 39 | 27 | 11 | 51 | 9 | 17 | 16 | 12 | 14 | 9 | 23 | 51 | 14.158 |
| 4 | 32 | 31 | 10 | 7 | 13 | 62 | 8 | 12 | 5 | 10 | 5 | 19 | 62 | 17.858 |
| 5 | 25 | 7 | 11 | 4 | 2 | 1 | 2 | 27 | 6 | 9 | 1 | 9.4 | 27 | 9.324 |
| 6 | 6 | 18 | 9 | 8 | 27 | 8 | 17 | 6 | 4 | 9 | 4 | 11.2 | 27 | 7.193 |
| 7 | 23 | 19 | 29 | 53 | 22 | 25 | 33 | 33 | 21 | 42 | 19 | 30 | 53 | 10.708 |
| 8 | 29 | 20 | 11 | 28 | 22 | 7 | 10 | 2 | 8 | 13 | 2 | 15 | 29 | 9.226 |
| 9 | 26 | 62 | 34 | 68 | 29 | 11 | 19 | 14 | 7 | 5 | 5 | 27.5 | 68 | 21.936 |
| 10 | 10 | 28 | 17 | 107 | 161 | 34 | 12 | 187 | 32 | 10 | 10 | 59.8 | 187 | 66.813 |
| Mean | 21.8 | 27.7 | 15.7 | 30.7 | 40.1 | 16.7 | 13.8 | 30.8 | 12 | 12.7 | | | | |
| SD | 9.31 | 15.94 | 10.64 | 35.54 | 46.37 | 18.87 | 8.73 | 55.77 | 9.17 | 10.69 | | | | |

SD = standard deviation.

Note: The number in each case is the number of thumbnail findings for that reader falling outside the gold standard target time range.

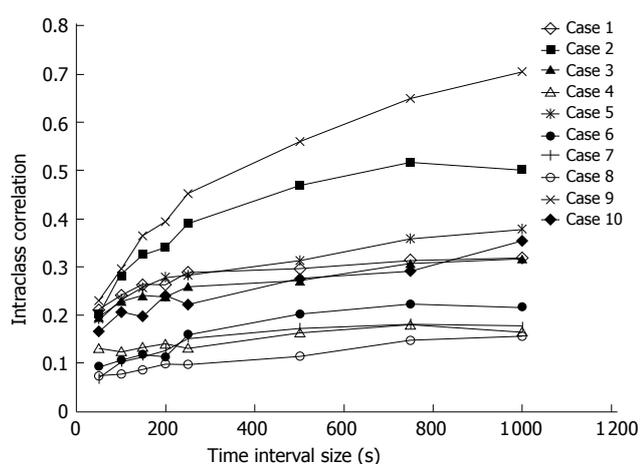


Figure 1 Inter-reader concordance analyzed separately by case.

analyses, the combination of 5 000 s and reader's threshold of 7/10 was the most optimal. This combination resulted in a reader's sensitivity of 100%, a low average incidental finding/false positive percentage and a low number of minutes viewing incidental finding/false positive time

interval video images. However, while this time interval was still relatively large (83 min), smaller time interval sizes had higher rates of errors and/or failed to yield 100% sensitivity.

DISCUSSION

Capsule endoscopy is a newly developed diagnostic modality that allows visualization of the entire small intestine. However, the process of selecting, training, and validating an individual's ability to accurately perform capsule endoscopy image analysis has not been well studied. Therefore, we performed a systemic study to compare and validate the capsule endoscopy readers' performance on capsule endoscopy image analysis. The goal of our study was to determine the sensitivity and incidental finding/false positive rate as well as the intraclass correlation of novice readers and to determine if the concept of analyzing the same capsule endoscopy recording by multiple novice readers was an effective and accurate approach for capsule endoscopy image interpretation.

We hypothesized that novice readers could reliably

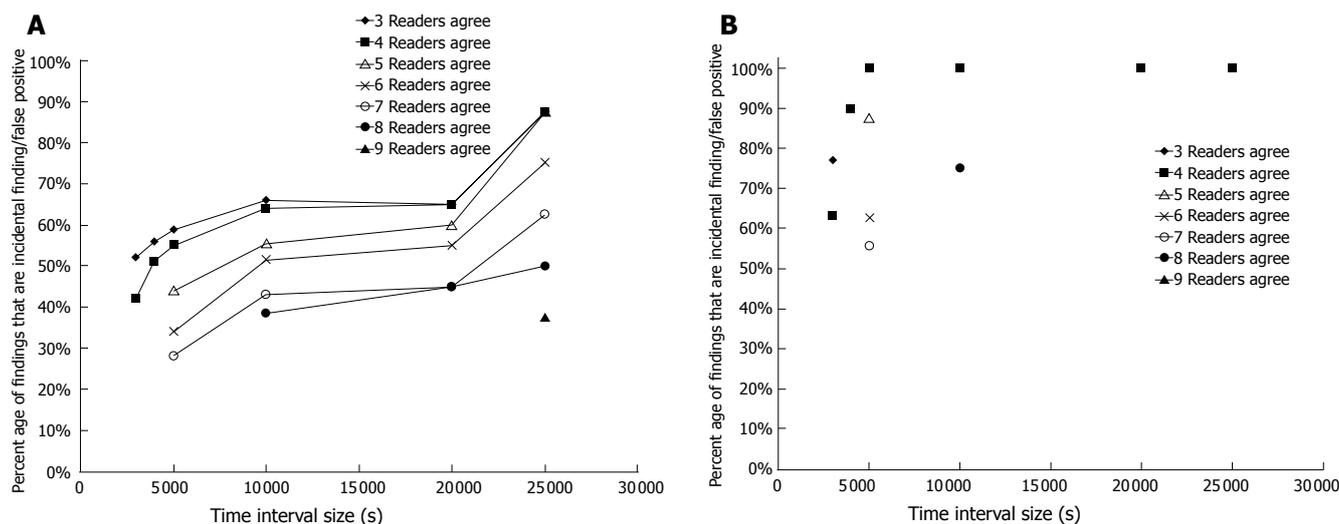


Figure 2 Average (A) and maximum (B) incidental finding/false positive rate across 10 cases by reader's threshold and time interval size.

detect small bowel lesions with a high sensitivity and a large number of incidental/false positive findings. In our study, each reader made a moderate number of incidental or false positive findings per recording. The following factors can help explain the moderate number of incidental or false positive findings: 1) the novice readers were asked to perform the capsule endoscopy analysis in a detailed, thorough and highly cautious fashion in an attempt to minimize the possibility of missing small bowel lesions; 2) the readers were untrained in interpreting capsule endoscopy images; 3) the “lodging” of capsule around the same spot in the small bowel caused some of the readers to thumbnail the same lesion several times; and 4) some of the incidental or false positive findings were small lesions, such as focal petechiae, areas of erythema or “mucosal breaks” with their clinical significance being still debatable.

We found that novice readers with minimal endoscopic experience were able to detect lesions on capsule endoscopy with a moderate-to-high sensitivity. Though the majority of the readers were unable to achieve 100% individual sensitivity, if we view the results by this panel of novice readers as a whole, every single gold standard target was detected. Therefore, perhaps the concept of analyzing the same capsule endoscopy recording by multiple novice readers may be an alternative yet effective and accurate method to interpret the capsule endoscopy images. This alternative approach might decrease the risk of having any lesions undetected by a single reader.

Inter-observer variability in analyzing capsule endoscopy recordings has been studied by Levinthal *et al.*¹⁴¹. The combined sensitivity of the group of novice readers from our study is comparable with the result achieved by Levinthal *et al.*¹⁴¹. In another published series, inter-observer variability was evaluated by comparing the interpretation results on 20 capsule endoscopy cases of an attending gastroenterologist and a 4th year therapeutic endoscopy student who has reviewed 15 capsule endoscopy cases prior to the participation of this study¹⁵¹. The authors found that there is a complete agreement between the two readers in 18/20 cases. Nonetheless, this

study only compared the clinically significant findings and did not report the number of incidental/false positive findings.

However, studies on inter-observer variability on capsule endoscopy interpretation are mostly documented in abstract forms. Hoffman *et al.*¹⁶¹ showed that physician extenders could save gastroenterologists' time in capsule endoscopy interpretation.

Analyzing capsule endoscopy recordings requires a significant time commitment from the gastroenterologist. As a result, a few studies have investigated the potential of using physician extenders to serve as screeners for interpreting capsule endoscopy images. The results from our study showed that novice readers could achieve a high sensitivity in capsule endoscopy analysis when their results were combined as a group. Therefore, to analyze the same capsule endoscopy recordings by multiple novice readers may be the most effective and accurate method for detecting all significant lesions on capsule endoscopy. This is especially important because some lesions may appear in a single frame and could be easily missed by a single reader. An analogy to this method is the airport luggage screening process, in which the luggage is screened through the X-ray/CT scanners, whereby the television screen is monitored by “highly trained” individuals who detect the “high risk items” (analogous to lesions). Suspicious bags are subsequently re-X-rayed and screened by several individuals and then high-risk items are manually inspected (analogous to endoscopy, push enteroscopy or surgical investigation).

Our study is the first systematic study to date addressing the issues of inter-observer variability in capsule endoscopy image analysis by a large group (>4 individuals) of readers. The most important clinical conclusion of our study is that a panel of novice readers with minimal endoscopic experience can detect small bowel lesions on capsule endoscopy recordings and pre-screen recordings to thumbnail potential abnormalities with a high sensitivity, allowing the gastroenterologists to review only the thumbnailed potential abnormalities. This concept serves as an alternative method to those proposed

in the previous studies (i.e. using gastroenterology students or endoscopy nurses). Furthermore, perhaps the most effective way to accurately detect all abnormalities on capsule endoscopy recordings is to analyze the same capsule endoscopy case by a number of readers. This approach to capsule endoscopy image analysis may decrease the number of medical errors. Our results suggest once again that physician extenders can serve as screeners for interpreting capsule endoscopy images and save a significant amount of time of the gastroenterologists and make capsule endoscopy more cost-effective and attractive to practising gastroenterologists. However, due to the moderate number of incidental/false positive findings, gastroenterologists must review these thumbnails to determine the clinical relevance of each finding. Future studies should also estimate the amount of time that gastroenterologists have to spend on the assessment of all the incidental and false positive findings by the physician extenders. Additional studies are ongoing to assess other reader cohorts' (endoscopy nurses, gastroenterology students, medical residents, non-medical personnel) abilities to detect abnormalities on capsule endoscopy before physician extenders begin to screen capsule endoscopy in everyday clinical practise.

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Serological pattern "anti-HBc alone": Characterization of 552 individuals and clinical significance

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Abstract

AIM: To investigate the prevalence and clinical significance of "anti-HBc alone" in an unselected population of patients and employees of a university hospital in southern Germany.

METHODS: All individuals with the pattern "anti-HBc alone" were registered over a time span of 82 mo. HBV-DNA was measured in serum and liver samples, and clinical charts were reviewed.

RESULTS: Five hundred and fifty two individuals were "anti-HBc alone" (of 3004 anti-HBc positive individuals; 18.4%), and this pattern affected males (20.5%) more often than females (15.3%; $P < 0.001$). HBV-DNA was detected in serum of 44 of 545 "anti-HBc alone" individuals (8.1%), and in paraffin embedded liver tissue in 16 of 39 patients tested (41.0%). There was no association between the detection of HBV genomes and the presence of biochemical, ultrasonic or histological signs of liver damage. Thirty-eight "anti-HBc alone" patients with cirrhosis or primary liver carcinoma had at least one additional risk factor. HCV-coinfection was present in 20.4% of all individuals with "anti-HBc alone" and was the only factor associated with a worse clinical outcome.

CONCLUSION: In an HBV low prevalence area, no evidence is found that HBV alone causes severe liver damage in individuals with "anti-HBc alone". Recommendations for the management of these individuals are given.

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Key words: HBV markers; HBV serology; Hepatitis B virus; Hepatocellular carcinoma; Occult HBV infection

INTRODUCTION

Antibodies to hepatitis B virus (HBV) core antigen (anti-HBc) are the most important marker of HBV infection. They are present when symptoms of hepatitis first appear and usually persist for life, irrespective if the infection resolves or remains chronic. Complete recovery from acute and chronic hepatitis B is associated with the loss of HBV surface antigen (HBsAg) and appearance of HBsAg-specific antibodies (anti-HBs) in serum. Thus, anti-HBc is usually accompanied by HBsAg or anti-HBs. However, the detection of "anti-HBc alone" (as the only marker of HBV infection) is not an infrequent serological pattern. In areas with low HBV prevalence (most parts of Europe and the United States) "anti-HBc alone" is found in 10-20% of all individuals with HBV markers, according to 1-4% of the general population^[1,2]. This pattern poses problems because it provides no exact diagnosis. It is seen in acute infections, in the interval between the loss of HBsAg and the appearance of anti-HBs, as well as in chronic and past infections. Some individuals with "anti-HBc alone" carry HBV in their serum, their proportion varies greatly between 0.2% in blood donors and 47% in iv drug abusers^[1].

The clinical significance of "anti-HBc alone" is greatly unknown. Longitudinal studies to explore the long-term clinical outcome are hardly practicable. We therefore investigated all individuals found positive for "anti-HBc alone" in our diagnostic laboratory over a period of 82 mo in regard to their virological and clinical findings.

MATERIALS AND METHODS

Study design

From July 1996 through April 2003, all individuals were registered whose sera tested reactive for anti-HBc and negative for HBsAg and anti-HBs for the first time. Our laboratory routinely received samples from patients and employees of a University hospital in Southern Germany.

Table 1 Distribution of HBV, HCV and HIV markers (tested with AxSYM™) in all individuals referred to the laboratory

| | HBsAg neg anti-HBs neg | Anti-HBc pos HBsAg pos Anti-HBs neg | HBsAg neg anti-HBs pos | Anti-HBc neg |
|-----------------------------------|---------------------------|---|---------------------------|----------------------|
| Study period | | 82 mo (6/96-4/03) | | 12 mo (1/03-12/03) |
| <i>n</i> (% of anti-HBc pos) | 552 (18.4 %) | 412 (13.7 %) | 2040 (67.9 %) | 4398 |
| Male/female (ratio) | 370/180 (2.06 : 1) | 251/145 (1.73 : 1) | 1185/851 (1.39 : 1) | 2631/1766 (1.49 : 1) |
| Age (yr; mean±SD) | 58.5±16.5 | 42.8±17.4 | 57.4±16.7 | 50.4±19.6 |
| Anti-HCV pos/ <i>n</i> tested (%) | 112/550 (20.36 %) | 20/323 (6.19 %) | 144/1983 (7.26 %) | 95/4285 (2.22 %) |
| Anti-HIV pos/ <i>n</i> tested (%) | 17/540 (3.15 %) | 8/206 (3.88 %) | 23/1571 (1.46 %) | 7/3768 (0.19 %) |

Totally 552 individuals (≥ 12 mo of age) with reactivity to “anti-HBc alone” were included in a database, and results of previous or follow-up testing for HBV markers were added when available for the study period. Clinical charts were reviewed to collect data about the medical history and clinical status of each individual.

Serological test

HBsAg, anti-HBs, anti-HBc, and anti-HBe were tested with microparticle enzyme immunoassays (AxSYM HBsAg™, AxSYM AUSAB™, AxSYM CORE™, AxSYM Anti-HBe™; Abbott Laboratories, Abbott Park, IL). Reactivity to “anti-HBc alone” (HBsAg and anti-HBs negative) was confirmed with a second microparticle enzyme immunoassay (IMx Core, Abbott, Delkenheim, Germany). When the new chemiluminescent microparticle immunoassay ARCHITECT anti-HBc™ (Abbott Laboratories, Abbott Park, IL) became available, all newly identified “anti-HBc alone” patients were additionally tested with this assay if there was enough serum available ($n=113$).

Antibodies against HCV and HIV were measured with microparticle enzyme immunoassays (AXSYM HCV™, AXSYM HIV-1/ HIV-2™; Abbott Laboratories, Abbott Park, IL). Positive test results were confirmed with Western blot assays Abbott MATRIX HCV 2.0 (Abbott Diagnostics, Wiesbaden-Dielkenheim, Germany) and NEW LAV BLOT I and II (BIORAD Laboratories, Munich, Germany), respectively.

PCR assays

HBV-DNA was isolated from serum with the QIAmp™ blood kit (Qiagen, Hilden, Germany). Paraffin embedded liver tissue was available for 39 patients from liver biopsies (hepatitis C, $n=16$; cirrhosis of the liver, $n=5$; liver abscess, $n=2$; deteriorating liver function after gastric perforation, $n=1$) or from liver surgery (liver transplantation for HCV associated cirrhosis, $n=4$; liver transplantation for alcohol associated cirrhosis, $n=3$; hepatocellular carcinoma, $n=4$; liver metastases, $n=2$; thrombosis of the portal vein, $n=1$; planned organ donation after brain death due to cerebral hemorrhage, $n=1$). Five to ten 5- μ m sections were deparaffined as described before^[3] and DNA was prepared using the QIAmp™ blood and tissue kit (Qiagen, Hilden, Germany). HBV-DNA was quantitatively measured using a kinetic fluorescence detection system (TaqMan PCR)^[4]. The lower detection limit of this assay was 50-100 genomic copies per mL.

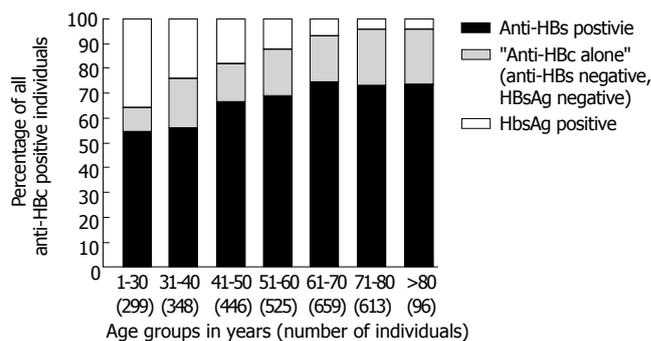


Figure 1 Distribution of HBV markers in 2986 anti-HBc positive individuals of different age groups, all referred to one virological laboratory over a period of 82 mo.

HCV-RNA in serum was detected using the Cobas AmpliCor 2, 0 (Roche Diagnostics, GmbH, Mannheim, Germany).

Statistical analysis

The SPSS program package version 10.0 (SPSS Inc., Chicago, IL) was utilized to perform Fisher's exact test, χ^2 test and *t*-test. The significance level was set at 5%, all *P*s resulted from two-sided tests.

RESULTS

HBV serology

From July 1996 through April 2003, 3004 individuals tested positive for anti-HBc (Table 1) and 552 showed reactivity to “anti-HBc alone” (18.4%). “Anti-HBc alone” was seen more often in males (370/1806; 20.5%) than in females (180/1176; 15.3%; $P<0.001$). The percentage of individuals positive for “anti-HBc alone” was highest in the age group 71-80 years (139/613; 22.7%) and lowest in the youngest group 1-30 years of age (28/299; 9.4%; Figure 1).

In 113 “anti-HBc alone” individuals, apart from the confirmatory test, an additional assay for anti-HBc was performed (ARCHITECT™): anti-HBc was positive in 111 individuals, negative in one individual, and alternating negative and positive in two consecutive samples from another individual.

HCV and HIV coinfection

One hundred and twelve of 550 “anti-HBc alone” individuals (20.4%) were positive for anti-HCV. HCV-RNA was detected in 98 of 108 anti-HCV positive patients tested (90.7%). Five hundred and forty “anti-HBc alone” individuals were tested for anti-HIV and 17 (3.1%) were positive.

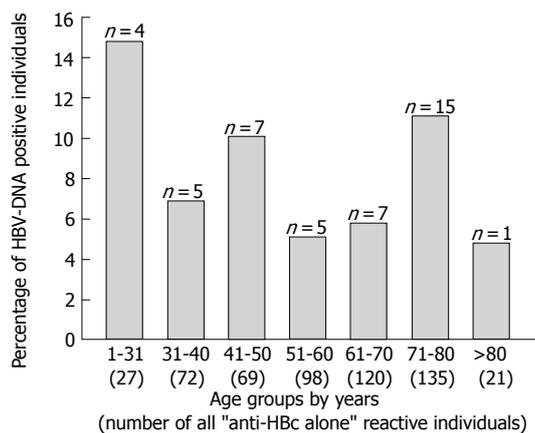


Figure 2 Percentage of HBV-DNA positive individuals (in serum) of all "anti-HBc alone" reactive individuals of different age groups.

Detection of HBV-DNA in serum and liver

Sera from 545 "anti-HBc alone" individuals were tested for the presence of HBV-DNA by PCR and 44 were positive (8.1%). The viral load was between 50 (lower limit of detection) and 1000 copies/ml serum. The percentage of HBV-DNA positive individuals was highest in the youngest group 1-30 years of age (4/27; 14.8%) and lowest in the age group > 80 years (1/21; 4.8%; Figure 2).

Paraffin embedded liver tissue was available from 39 patients. HBV-DNA was detected in 16 (41.0%): in 12 of 32 liver samples (37.5%) which had originally been taken because a chronic liver damage was suspected, and in 4 of 7 liver samples (57.1%) taken for other reasons. Considering the results of the histological examination, HBV-DNA was detected in 2 of 17 livers with cirrhosis, in 3 of 9 primary liver cancers and in 11 of 13 livers without cirrhosis or primary carcinoma.

Only 2 of the 39 patients with available liver samples were HBV-DNA positive in serum: one patient with chronic hepatitis C (no cirrhosis or carcinoma) was HBV-DNA positive both in serum and liver, and one patient with hepatocellular carcinoma was HBV-DNA positive only in serum but not in liver. Thus, liver HBV-DNA was positive in 1 of 2 patients with serum HBV-DNA and in 15 of 37 patients without serum HBV-DNA.

Clinical presentation

The 552 "anti-HBc alone" individuals were initially tested for HBV markers due to the following reasons: routine screening prior to invasive procedures ($n=416$); diagnostic evaluation of cirrhosis of the liver ($n=20$), hepatocellular carcinoma ($n=11$), or elevation of transaminases ($n=10$); diagnostic evaluation of a known HCV ($n=28$) or HIV ($n=16$) infection; routine screening of health care workers ($n=6$); follow up evaluation of a known hepatitis B infection ($n=4$); and evaluation of a needle stick injury recipient ($n=3$). No diagnosis or cause of testing was given in 38 cases. In 90 patients (16.3%), the positive anti-HBc status was known prior to the actual investigation. Thirteen patients (2.4%) had suffered from jaundice of unknown origin earlier in their life. Information about suspected modes of infection or risk factors was available for 141 patients (25.5%): origin from an HBV hyperendemic area

($n=95$), intravenous drug abuse ($n=21$), HIV-infection ($n=17$), previous blood transfusion or transplantation ($n=17$), health care worker ($n=10$), and HBV-positive sex partner ($n=4$). Twenty individuals had more than one of these risk factors.

Alanine transaminase (ALT) activity was measured in 445 individuals and was normal in 279, up to twofold elevated in 89 and more than twofold elevated in 77 patients. Ultrasonic investigation of the liver was performed in 237 patients and revealed enlargement/steatosis of the liver in 69, signs of cirrhosis in 20, liver carcinoma in 10, and was non-distinctive in 20 patients. Histological examination of the liver was performed in 43 patients and showed cirrhosis in 16, hepatocellular carcinoma with cirrhosis in 10, active viral hepatitis in 8 (all with chronic HCV infection, HBV-DNA in serum positive in 1/8), steatosis of the liver in 3, liver abscess in 2, metastases of colon carcinoma in 2, primary biliary cirrhosis in one, and combined hepatocellular-cholangiocarcinoma without cirrhosis in one patient.

Combining all results of clinical, ultrasonic and histological examinations, we identified 38 "anti-HBc alone" patients with severe chronic liver damage (cirrhosis of the liver, $n=27$; hepatocellular carcinoma, $n=10$; combined hepatocellular-cholangiocarcinoma, $n=1$). All 38 patients had at least one additional risk factor capable of causing the severe liver damage: chronic HCV infection ($n=18$), alcohol abuse ($n=14$), combined alcohol abuse and HCV infection ($n=5$), and non-alcoholic steatohepatitis ($n=1$).

"Anti-HBc alone" reactive individuals with positive and negative HBV-DNA in serum were compared regarding sex, age, ALT level, liver pathology, anti-HBs-titer, anti-HBe-status, HCV- and HIV coinfection. Statistically significant differences were not found (Table 2).

In those patients investigated, the detection of anti-HCV antibodies was associated with an elevation of ALT activity ($P<0.001$) and with the presence of cirrhosis or primary carcinoma of the liver (Table 3).

Serological and clinical course

In 151 of the 552 "anti-HBc alone" patients (27.4%), more than one serum was investigated during the study period. In 111 individuals, reactivity to "anti-HBc alone" was confirmed by subsequent testing after a mean follow up period of 17.1 mo (minimum-maximum, 0-77 mo). Eleven individuals were anti-HBs positive (16.8 ± 8.4 IU/L) when retested after a mean of 11.5 months (1-38 mo), and 10 individuals had anti-HBs titers fluctuating slightly above and below 10 IU/L over a period of 44.9 mo (10-82 mo).

One "anti-HBc alone" patient (male, 45 years, HBV-DNA in serum positive) showed HBV reactivation with reappearance of HBsAg, HBeAg, increase of HBV-DNA and loss of anti-HBs - but normal ALT activity - after treatment with rituximab (humanized monoclonal antibody to CD 20) and chemotherapy (cyclophosphamide, doxorubicin, vincristine, prednisone) for non-Hodgkin lymphoma.

For 18 patients, a previous serum sample was available not showing the "anti-HBc alone" pattern. Twelve patients were anti-HBs positive (16.6 ± 4.7 IU/L) at an average of

Table 2 Clinical data in individuals with reactivity to "anti-HBc alone"

| | | HBV-DNA-PCR in serum ¹ | |
|-------------------------------|----------------------------|-----------------------------------|-----------------|
| | | Positive | Negative |
| | <i>n</i> | 44 | 501 |
| | Males/females ² | 27/17 | 336/163 |
| | Age (mean±SD) | 57.2±18.6 | 58.5±16.2 |
| ALT ² | Normal | 23/35 (65.7%) | 251/402 (62.4%) |
| | ≤ Twofold increase | 7/35 (20.0%) | 81/402 (20.1%) |
| | > Twofold increase | 5/35 (14.3%) | 70/402 (17.4%) |
| Liver ultrasound ² | no pathological findings | 5/11 (45.5%) | 112/204 (54.9%) |
| | Steatosis | 5/11 (45.5%) | 63/204 (30.9%) |
| | Cirrhosis | 0/11 | 20/204 (9.8%) |
| | Carcinoma | 1/11 (9.1%) | 9/204 (4.4%) |
| Liver histology ² | No pathological findings | 0/2 | 5/42 (11.9%) |
| | Viral inflammation | 1/2 (50%) | 7/42 (16.7%) |
| | Steatosis | 0/2 | 3/42 (7.1%) |
| | Cirrhosis | 0/2 | 17/42 (40.5%) |
| | Carcinoma | 1/2 (50%) | 10/42 (23.8%) |
| | Anti-HBs titer (IU/L) | 1.9±2.5 | 2.6±3.0 |
| Anti-HBe pos/ <i>n</i> tested | 6/9 (66.7%) | 53/138 (38.4%) | |
| Anti-HCV pos/ <i>n</i> tested | 7/44 (15.9%) | 103/500 (20.6%) | |
| Anti-HIV pos/ <i>n</i> tested | 0/43 (0%) | 17/491 (3.5%) | |

¹ There were no statistically significant differences between "anti-HBc alone" individuals with positive and negative HBV-DNA in serum regarding all parameters listed in Table 2.

² Missing numbers - not available/ not tested.

31.3 mo (1-59 mo) before being detected as "anti-HBc alone".

Six patients were previously HBsAg positive. Three of them had an acute HBV infection recently: (1) one female patient (14 years old) lost HBsAg after 9 mo, but remained HBV-DNA positive and anti-HBs negative during an additional 34 mo; (2) one male patient (45 years old) was "anti-HBc alone" (HBV-DNA positive) 4 mo after the acute infection and tested anti-HBs-positive after additional 4 mo; (3) one male patient (17 years old) was "anti-HBc alone" (HBV-DNA negative) 4 mo after the acute infection and was anti-HBs positive when retested after an additional 10 mo. Two patients just cleared a chronic HBV infection: one male patient (37 years old) had a chronic HBV and HCV infection for at least 6 years and cleared HBsAg (but not HBV-DNA or HCV-RNA) after treatment with PEG-interferon and ribavirin; the other male patient (59 years old) was discovered to be HBsAg positive by routine screening (ALT normal, HBV-DNA negative), and was "anti-HBc alone" when retested after 5 mo. The sixth patient (male, 41 years old) showed reverse seroconversion (reappearance of HBsAg and loss of anti-HBs) and moderate ALT elevations 14 mo after allogeneic hematopoietic stem cell transplantation^[5]. He lost HBsAg again after 4 mo and had alternating anti-HBs positive and negative ("anti-HBc alone") but HBV-DNA negative during follow up (53 mo).

DISCUSSION

The present study has primarily been undertaken to assess the clinical significance of the serological pattern "anti-HBc alone". Over a period of 6 years and 8 mo,

Table 3 Clinical data in "anti-HBc alone" individuals and anti-HCV status

| | | Anti-HCV | | P-value |
|-------------------------------|--------------------------|----------|----------|-----------------|
| | | Positive | Negative | |
| Sex | Males | 76 | 290 | NS ² |
| | Females | 36 | 141 | |
| ALT ¹ | Normal | 31 | 244 | <0.001 |
| | ≤ Twofold increase | 20 | 67 | |
| | > Twofold increase | 48 | 29 | |
| Liver ultrasound ¹ | No pathological findings | 27 | 89 | 0.03 |
| | Steatosis | 25 | 43 | |
| | Cirrhosis | 11 | 9 | |
| | Carcinoma | 6 | 4 | |
| Liver histology ¹ | No pathological findings | 0 | 0 | <0.001 |
| | Viral inflammation | 8 | 0 | |
| | Steatosis | 2 | 1 | |
| | Cirrhosis | 12 | 4 | |
| | Carcinoma | 6 | 4 | |

¹ Missing numbers: not available/ not tested.

² NS: not significant.

552 individuals with this pattern were identified in our laboratory, accounting for 18.4% of all individuals with positive anti-HBc. Forty-four of 545 "anti-HBc alone" patients had circulating HBV-DNA with less than 1000 copies/ml. Depending on the sensitivity of the PCR assay, the proportion of HBV-DNA carriers in this study (8.1%; detection limit 50 HBV-DNA-copies/ml) complies with other studies reporting carrier rates of 3.3% and 7.7% (detection limits of 5000 and 100 HBV-DNA copies/mL, respectively) in unselected German individuals or blood donors^[2,6]. Even more "anti-HBc alone" individuals with very low viremia would probably be found if the sensitivity of the assay were increased. In paraffin embedded liver tissue, we detected HBV-DNA in 16/39 "anti-HBc alone" patients (41.0%), only one of them was also PCR positive in serum. Thus, a significant fraction of "anti-HBc alone" individuals carry HBV genomes. However, HBV-DNA has also been detected in individuals with anti-HBs antibodies. Two German studies have reported a total of six HBV-DNA-positive blood donors with anti-HBs levels greater than 100 IU/L^[7,8]. After the resolution of an HBV infection, a periodical release of virions from hepatocytes, as first described by Reherman *et al*^[9], can occur in HBsAg-negative, anti-HBc positive individuals, although more often in persons who lack anti-HBs compared to those with detectable anti-HBs^[10].

The essential question about these "occult" infections applies to their clinical impact. Since HBV-DNA has been found in serum and liver samples of HBsAg-negative patients with cirrhosis or primary liver cancer^[11-13], it has been suggested that "anti-HBc alone" individuals may suffer from chronic liver injury with the associated oncogenic potential. Herein, we identified 38 "anti-HBc alone" patients with severe chronic liver damage. All 38 patients had at least one other risk factor which could cause the liver damage by itself. In addition, HBV-DNA was less often detected in the liver of these patients (2/17 with cirrhosis, 3/9 with primary carcinoma) than in liver samples from patients without chronic liver damage

(11/13). When all “anti-HBc alone” individuals of this study were analyzed, there was no association between the detection of HBV-DNA in serum and ultrasonic, histological or biochemical evidence of liver damage. Therefore, the present study provides no evidence that HBV alone causes liver damage in “anti-HBc alone” individuals.

Although an occult HBV infection in “anti-HBc alone” individuals seems to be inoffensive, it might become injurious in the case of immunosuppression leading to viral reactivation. In patients with past infection (anti-HBs and anti-HBc positive), HBV reactivation has been reported during chemotherapy, HIV infection, and after kidney and bone marrow transplantation^[14-17]. In the present study, HBV reactivation with reappearance of HBsAg was observed in one “anti-HBc alone” patient after treatment with rituximab (humanized monoclonal antibody to CD 20) and chemotherapy for non-Hodgkin lymphoma. This patient had no clinical hepatitis, but there are at least two reported cases of HBV reactivation with liver inflammation after rituximab and chemotherapy: one patient died from liver failure^[18], and the other reactivation occurred in a patient with preexisting anti-HBs and anti-HBc antibodies^[19]. Our findings justify the cautious use of rituximab in “anti-HBc alone” patients and emphasize that these patients carry the risk for HBV reactivation during immunosuppression in general.

When a patient is diagnosed to be reactive for “anti-HBc alone”, several explanations might apply to this phenomenon. First, a certain proportion of “anti-HBc alone” individuals will be false positive, depending both on the anti-HBc test used and on the HBV prevalence of the population investigated. We aimed to minimize false positives by including only individuals who were anti-HBc positive in a second confirmatory assay. When a subset of 113 individuals was investigated with a third anti-HBc test, reactivity was confirmed in 111 (98%). Although all three assays were from the same manufacturer, we estimated that the population was truly anti-HBc reactive. An alternative assay from a different manufacturer might provide a better confirmation, but there was no serum for additional assays available.

Second, “anti-HBc alone” individuals might be in the window phase of an acute HBV infection when HBsAg disappears followed by anti-HBs a few weeks later. Herein, only 6 of 552 “anti-HBc alone” individuals were known to be HBsAg positive before: three were in the window period after an acute infection, two probably just cleared a chronic infection, and one lost HBsAg again after HBV reactivation following bone marrow transplantation. In a low HBV prevalence area as in Germany, the proportion of “anti-HBc alone” individuals in the window period is very low, but individuals in this period are probably infectious.

Third, “anti-HBc” alone can also reflect an HBV infection which has resolved many years or decades earlier. In the majority of our patients, reactivity for anti-HBc was an incidental finding, most were healthy and not aware of a previous liver inflammation. In 151 of the 552 “anti-HBc alone” individuals, more than one sample was investigated during the study period: 12 individuals were anti-HBs

positive before being detected as “anti-HBc alone”, 10 individuals had anti-HBs titers fluctuating around 10 IU/L during follow up, and 11 individuals were weakly anti-HBs positive when retested. These findings suggest that a significant proportion of “anti-HBc alone” individuals are comparable to those still showing anti-HBs.

Fourth, a large fraction of “anti-HBc alone” patients is assumed to have an unresolved chronic infection with low grade, possibly intermittent virus production. These individuals have detectable HBV-DNA in serum (8.1% in this study) and are potentially infectious. The negative HBsAg assay is unclear in these cases, but can be caused by very low concentrations of HBsAg, fixation of HBsAg in immune complexes, or by HBsAg mutations^[20,21]. From a practical point of view, there seems to be no clear-cut distinction between “late immunity” and “unresolved infection”: HBV-DNA has also been detected in anti-HBs positive individuals, and HBV reactivation can occur both in anti-HBs positive and in “anti-HBc alone” patients. In the present study, there was no difference in the clinical picture between HBV-DNA negative and positive individuals with “anti-HBc alone”.

One final explanation for the phenomenon “anti-HBc alone” is the suppression of HBV replication by an HCV coinfection. In the present study, 112/550 “anti-HBc alone” individuals (20.4%) were coinfecting with HCV, and patients with HCV coinfection more often had an elevated ALT activity or chronic liver damage. The detrimental effect of HCV coinfection has previously been reported in HBsAg positive patients with chronic HBV infection^[22,23].

In conclusion, we performed an extensive study including 552 “anti-HBc alone” individuals of all age groups and found no single patient who had severe liver damage and no additional risk factor other than the “anti-HBc alone” pattern. There was also no association between the detection of HBV genomes in serum and/or liver and damage of the liver. Therefore, the probability that HBV alone causes severe liver damage in “anti-HBc alone” patients seems to be low.

This study supports suggestions for the practical management of “anti-HBc alone” individuals which have previously been made by a group of experts^[1]: When this pattern is observed for the first time, a false positive anti-HBc reactivity should be ruled out by a second anti-HBc test, preferably an assay with a different format. In some cases, anti-HBe will also be present and confirm the authenticity of the hepatitis B markers. All “anti-HBc alone” individuals must be screened for an HCV coinfection. To search for a chronic HBV infection (or an infection in the window phase), viral DNA should be measured with the most sensitive DNA amplification method (capable of detecting ≤ 100 genomes per mL), and the ALT activity should be determined. It appears sufficient to re-evaluate individuals without HBV-DNA and with normal ALT level after a longer period of time, e.g. every five years. In case of a relevant ALT elevation, a liver biopsy seems appropriate to reach a conclusion concerning therapy. Antiviral treatment should be considered if HBV-DNA and biochemical and histological signs of active hepatitis are present. Since “anti-HBc alone” patients with this constellation are rare, they should be monitored by an

experienced hepatologist. Individuals with positive HBV-DNA PCR and normal ALT values should be assessed in yearly intervals. When "anti-HBc alone" individuals are subjected to severe immunosuppression (e.g. during anti-tumor chemotherapy or transplantation), HBV-DNA assays should be applied to recognize viral reactivation. Individuals with "anti-HBc alone" must be advised not to donate blood.

Further research is needed to answer open questions about the pattern "anti-HBc alone", e.g. concerning the reason for the male predominance and the infectivity of affected individuals.

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Hypertriglyceridemia is positively correlated with the development of colorectal tubular adenoma in Japanese men

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Abstract

AIM: To determine the real association between serum lipid levels and colonic polyp formation.

METHODS: We performed a large scale retrospective study to analyze the correlation between the incidence of colorectal adenoma or carcinoma and the fasting serum levels of total cholesterol (TC) and triglycerides (TG) in patients who underwent total colonoscopy for screening for colon cancer.

RESULTS: Both levels were significantly elevated in patients with adenomas as compared with patients without any neoplastic lesion (TC 207.6 ± 29.5 vs 199.5 ± 34.3 , $n=4883$, $P<0.001$; TG 135.0 ± 82.2 vs 108.7 ± 71.5 , $n=4874$, $P<0.001$). The difference was significant in patients with tubular adenoma but not in those with villous or serrated adenoma. Multiple logistic regression analysis including age and sex revealed that TG was an independent correlation factor in male ($P<0.01$), but not in female patients. The level of TG in patients with invasive carcinoma did not show a significant elevation from that in patients with adenoma. These findings suggest that hypertriglyceridemia is an independent risk factor for colonic adenoma in men.

CONCLUSION: Although a high level of serum triglyceride does not appear to be mechanically involved in the development of carcinoma, reduction of serum TG and intensive surveillance with total colonoscopy may have benefit in men with hypertriglyceridemia.

INTRODUCTION

Many studies have suggested that a high intake of dietary fat tends to promote colon carcinogenesis^[1-4]. Regarding serum lipid levels, early studies showed an inverse association between cholesterol levels and colon cancer, whereas many contradictory results have also been reported later^[5-8]. These findings suggest that the association of low serum cholesterol with malignancies is a consequence of the impaired nutritional status rather than a cause of cancer.

As compared with total cholesterol, less information is available on the relationship between serum triglyceride and colorectal neoplasm. The striking similarity of lifestyle risk factors for colorectal cancer and insulin resistance suggests that hypertriglyceridemia as well as hyperinsulinemia and hyperglycemia associated with insulin resistance support the development of colon cancer^[9-12]. Moreover, recent animal studies showed direct evidence suggesting a positive effect of triglyceride on the development of aberrant crypt foci^[13] and adenomatous polyps^[14,15]. This suggests overlapping mechanisms that increase the risk of colon cancer and hyperlipidemia. On the contrary, other reports failed to detect a significant association between serum levels of triglyceride as well as cholesterol and risk of colorectal cancer in human^[16,17] and animal studies^[18].

Thus, the true association between circulating lipid levels and colonic neoplasm development has not been satisfactorily determined in humans. In our clinic, we have performed a large number of total colonoscopies for screening for colon cancer, and intensively attempted to find colonic adenomatous polyps, since it is generally accepted that most colorectal cancers arise from adenomatous polyps^[19]. From our experience, we retrospectively analyzed the relationship between serum lipid levels and the presence of colonic adenoma as well as carcinoma.

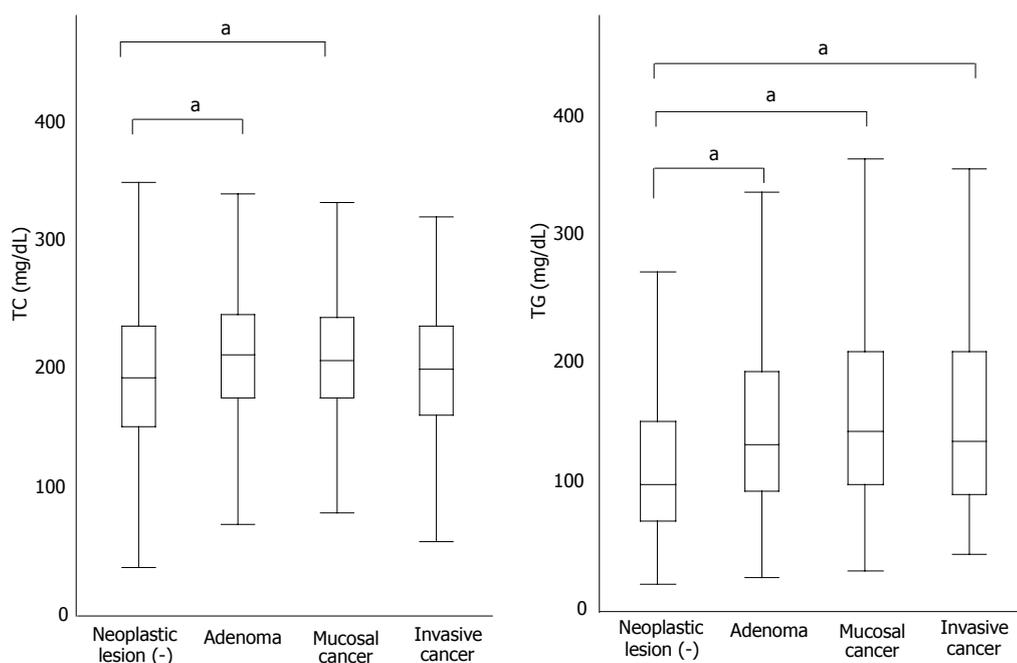


Figure 1 The average mean \pm SD of total cholesterol (TC) and triglyceride (TG) of patients with adenoma without cancer ($n=3821$), with adenoma as well as mucosal cancer ($n=386$), with invasive cancer as well as adenoma (110), and without any neoplastic lesions ($n=956$); a means P value less than 0.05 by ANOVA.

MATERIALS AND METHODS

Patients

A total of 4887 patients underwent total colonoscopy to screen for colon cancer from 1995 to 2003 in our clinic. The entire colon was carefully examined with a high resolution magnifying chromo-endoscope. More than 30 min was spent performing each colonoscopy, in order to avoid overlooking small lesions. All the adenomatous polyps including mucosal cancers were simultaneously resected by endoscopic polypectomy technique and examined histopathologically. Invasive cancer was found in 110 (2.3%) of 4887 patients even without subjective complaints. All the patients also had 1 to 77 adenomatous polyps in other parts of the colon. In the remaining 4777 patients, no adenomas were found in 956 (19.6%) patients, whereas at least one adenoma could be detected in the other 3821 (78.2%) patients, and all of them were endoscopically removed simultaneously. In 386 cases, 1 to 3 adenomas were histologically diagnosed to contain mucosal carcinoma. In these 3931 patients with adenomas (including patients with invasive cancer), only one adenoma was detected in 660 (13.5%) patients, 2 in 549 (11.2%) and 3 in 458 (9.4%) patients. In the other patients, 1934 (39.6%) had 4 to 9 adenomas, while the other 788 (16.1%) patients had 10 or more adenomas in the entire colon. Histological examination revealed mucosal cancer in 386 (9.9%) patients and that most of the resected adenomas were diagnosed as tubular adenoma, and that tubular adenoma was detected in 3928 (80%) of the total patients. Villous adenoma and serrated adenoma were observed in 53 (1.4%) and 36 (0.9%) cases, patients.

Determination of serum cholesterol and triglycerides

The fasting serum levels of two major lipids, total cholesterol (TC) and triglycerides (TG), were measured just before colonoscopy in 4883 and 4874 cases, respectively. Then, the relationship between the incidence of colonic

neoplasm and fasting serum levels of these lipids was retrospectively evaluated.

Statistical analysis

Statistical analysis was performed with JUMP software. The serum lipid levels in patients were analyzed by ANOVA and Fisher's exact test. The odds ratios were evaluated by multiple logistic regression tests. Values of P less than 0.05 were considered statistically significant.

RESULTS

Serum levels of TC and TG in patients with tubular, villous and serrated adenomas

The serum levels of TC and TG in patients with adenoma versus those with mucosal or invasive cancer showed a significant difference by ANOVA (Figure 1). Both values were significantly elevated in patients with adenomas as compared with patients without any neoplastic lesions (TC 207.6 ± 29.5 vs 199.5 ± 34.3 , $P < 0.001$; TG 135.0 ± 82.2 vs 108.7 ± 71.5 , $P < 0.001$). Patients with invasive or mucosal cancer showed very similar levels of TG (142.0 ± 75.1 , 139.3 ± 91.6 , respectively) to those with adenomas, which were significantly higher than that in patients without adenomas ($P < 0.001$). For TC, however, patients with mucosal cancer also showed a very similar level to those with adenomas (206.2 ± 28.7), while patients with invasive cancer showed a relatively reduced level (200.8 ± 28.3), which was similar to that in patients without neoplastic lesions. LDL cholesterol was examined in 1483 patients and showed a similar profile to TC (data not shown).

Next, we examined the difference in these lipid levels in patients with adenomas of different histology. As shown in Table 1, the serum levels of these lipids were significantly different in patients with tubular adenomas, but not in patients with villous or serrated adenomas. This indicates that hyperlipidemia has a specific association

Table 1 Serum levels of Total cholesterol (TC) and Triglyceride (TG) in patients with tubular, villous and serrated adenomas

| Pathological finding | TC | n | P value | TG | n | P value |
|----------------------|------------|------|------------------|-------------|------|------------------|
| tubular adenoma | | | | | | |
| absent | 199.6±34.4 | 958 | | 108.9±71.0 | 954 | |
| present | 207.3±29.3 | 3925 | <i>P</i> <0.0001 | 135.8±81.8 | 3920 | <i>P</i> <0.0001 |
| villous adenoma | | | | | | |
| absent | 205.8±30.6 | 4831 | | 130.5±80.7 | 4821 | |
| present | 211.5±24.6 | 52 | NS | 132.0±60.8 | 53 | NS |
| serrated adenoma | | | | | | |
| absent | 205.8±30.6 | 4848 | | 130.4±80.3 | 4838 | |
| present | 208.8±24.1 | 35 | NS | 149.3±102.3 | 36 | NS |

NS: not significant.

Table 2 Univariate analysis of risk factors for tubular adenoma

| Tubular adenoma | Total | Negative (%) | Positive (%) | P value |
|----------------------|-------|--------------|--------------|---------|
| Age (yr) | | | | |
| 10-56 | 2475 | 765 (30.9) | 1710 (69.1) | |
| 57-94 | 2412 | 194 (8.0) | 2218 (92.0) | <0.0001 |
| Gender | | | | |
| Male | 2997 | 402 (13.4) | 2595 (86.6) | |
| Female | 1890 | 557 (29.5) | 1333 (70.5) | <0.0001 |
| Hypercholesterolemia | | | | |
| Negative | 3318 | 695 (21.0) | 2623 (79.0) | |
| Positive | 1565 | 263 (16.8) | 1302 (83.2) | <0.0001 |
| Unknown | 4 | | | |
| Hypertriglyceridemia | | | | |
| Negative | 3488 | 784 (22.5) | 2704 (77.5) | |
| Positive | 1386 | 170 (12.3) | 1216 (87.7) | <0.0001 |
| Unknown | 13 | | | |

Hypercholesterolemia and hypertriglyceridemia were defined as total cholesterol>220mg/dL and TG>150 mg/dL, respectively.

Table 3 Adjusted odds ratios and 95% CIs for tubular adenoma for a change of 1SD in male or female patients

| | TC | | | TG | | |
|--------------------------------------|------|-----------|---------|------|-----------|---------|
| | OR | 95% CI | P value | OR | 95% CI | P value |
| Total (3787) | | | | | | |
| Gross OR | 1.3 | 1.21-1.39 | <0.0001 | 1.64 | 1.48-1.82 | <0.0001 |
| Age adjusted OR | 1.01 | 0.93-1.09 | 0.79 | 1.34 | 1.21-1.48 | <0.0001 |
| Age, gender and TC or TG adjusted OR | 1.04 | 0.96-1.13 | 0.334 | 1.13 | 1.01-1.09 | 0.013 |
| Male (2997) | | | | | | |
| Gross OR | 1.32 | 1.19-1.47 | <0.0001 | 1.35 | 1.19-1.56 | <0.0001 |
| Age adjusted OR | 1.09 | 0.98-1.23 | 0.13 | 1.24 | 1.09-1.43 | 0.0015 |
| Age and TC or TG adjusted OR | 1.04 | 0.91-1.07 | 0.555 | 1.23 | 1.07-1.42 | 0.0041 |
| Female (1890) | | | | | | |
| Gross OR | 1.49 | 1.34-1.65 | <0.0001 | 1.42 | 1.25-1.64 | <0.0001 |
| Age adjusted OR | 1.08 | 0.96-1.21 | 0.21 | 1.04 | 0.92-1.17 | 0.56 |
| Age and TC or TG adjusted OR | 1.06 | 0.95-1.02 | 0.29 | 1.02 | 0.91-1.16 | 0.7 |

with the development of tubular adenoma, but not the development of carcinomas or other types of adenomas.

Analysis of risk factors for tubular adenomas

We then performed multivariate analysis to determine the real association between serum lipid levels and tubular adenomas. Since univariate analysis showed that male and aged patients had colonic adenomas more frequently than their counterparts, we separately calculated the odds ratios for a change of one standard deviation (SD) adjusted for age in male and female patients (Tables 2 and 3). Multiple logistic regression analysis showed a clear contrast between TC and TG. When age and sex were considered, TC lost the independent relationship with adenoma, possibly because of the strong association with age. In contrast, TG was determined to be an independent correlation factor with tubular adenoma in total (*P*<0.05) and male (*P*<0.01) patients, but not in female patients.

DISCUSSION

Epidemiologic studies have suggested that a high fat intake is strongly related to the development of colorectal cancer, although it is unclear whether serum lipid levels

can predict the high risk population for colon cancer. Our results clearly indicate that hyperlipidemia is associated with the development of adenomatous polyps with tubular histology in the colon. Although a positive association between hyperlipidemia and colonic neoplasm has already been reported, this large scale study revealed two additional findings.

The first is that the serum levels of cholesterol and triglycerides are increased in patients with adenoma compared to those without adenoma, while serum levels in patients with cancer are not elevated compared to those with adenoma. This indicates that a high fat intake that increases serum lipid levels may play a role in the development of adenoma but not in the development of carcinoma from adenoma.

The other important finding is that TG, but not TC, was independently correlated with the development of tubular adenoma, and the association was observed only in male but not in female patients. Recent studies have demonstrated that APC-deficient FAP model mice showed a hyperlipidemic state, possibly due to reduced activity of lipoprotein lipase, and that systemic administration of pioglitazone, a PPAR ligand, to the mice markedly reduced the serum level of triglycerides as well as intestinal polyp

formation^[14,15]. These results are in agreement with our findings, and suggest the possibility that pioglitazone may be clinically effective to prevent polyp formation in humans.

The reason that the association was evident in male, but not in female patients, is yet unknown. The triglyceride level was significantly lower in women than in men in our series (106.17 ± 63.00 vs 145.90 ± 86.39 , $P < 0.001$), and tubular adenoma was observed in 70.5% of female patients versus 86.6% of male patients ($P < 0.001$). Since it was reported that the usage of estrogen reduced the incidence of colon adenoma as well as carcinoma by about 30%, resulting in reduced colon cancer-related mortality^[20], hormonal effects may be mechanically involved in the suppression of adenoma formation in the colon.

In summary, hypertriglyceridemia was shown to be an independent risk factor for colonic adenoma in men, although a high level of serum triglyceride did not appear to be directly involved in the development of colon carcinoma. Since all the adenomas were exclusively polypectomied in this clinic, no invasive cancers were detected at annual follow-up of those patients. Reduction of serum TG and intensive surveillance with total colonoscopy may be effective to prevent colon cancer in men with hypertriglyceridemia.

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Triple therapy of interferon and ribavirin with zinc supplementation for patients with chronic hepatitis C: A randomized controlled clinical trial

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Abstract

AIM: To study the therapeutic effect of interferon (IFN) and ribavirin with zinc supplement on patients with chronic hepatitis C viral (HCV) infection.

METHODS: A total of 102 patients confirmed histologically to have chronic HCV infection with genotype 1b and more than 100 KIU/mL of HCV were randomly assigned to each arm of the study and each received 10 million units of pegylated interferon (IFN-alpha-2b) daily for 4 wk followed by the same dose every other day for 20 wk plus ribavirin (600 or 800 mg/d depending on body weight), with or without polaprezinc (150 mg/d) orally for 24 wk. The primary endpoint was sustained virological response (SVR) defined as negative HCV-RNA in the serum 6 mo after treatment.

RESULTS: There were no differences in the clinical background between the two groups except for more females in the dual therapy group than in the other group ($P < 0.05$). SVR was observed in 33.3% of the triple therapy group and 33.3% of the dual therapy group. The side effects were almost the same in both groups except for gastrointestinal symptoms, which were less in the triple therapy group ($P = 0.019$).

CONCLUSION: Considered together, triple therapy of zinc plus IFN and ribavirin for HCV infection patients with genotype 1b and high viral load is not better than dual therapy except for lower incidence of gastrointestinal side effects.

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Key words: HCV; Zinc; Interferon; Ribavirin

INTRODUCTION

Chronic hepatitis C is a major cause of liver cirrhosis and hepatocellular carcinoma. It has become the most common indication for liver transplantation in many centers^[1,2]. Improvement of antiviral therapy for patients with chronic HCV infection has been recently achieved by the introduction of pegylated interferon (IFN-alpha-2b) combined with ribavirin^[3-5]. Because the sustained virological response rate in patients with HCV genotype 1b and high viral load is still insufficient, the search continues for new treatment strategies.

On the other hand, patients with chronic liver disease exhibit metabolic imbalances of trace elements such as high levels of iron and copper, and low levels of zinc, selenium, phosphorus, calcium and magnesium^[6]. Of these trace elements, zinc is a constituent of a number of enzymes involved in a large number of metabolic processes. Mild-to-severe zinc deficiency disturbs several biological functions such as gene expression, protein synthesis, immunity, skeletal growth and maturation, gonad development, pregnancy outcomes, taste perception and appetite^[7,8]. Zinc has been studied for its antiviral effects on HIV^[9], rhinovirus^[10] and herpes virus^[11]. In addition to its anti-inflammatory effects, zinc has an antioxidant effect^[12] and induces metallothionein (MT) which has radical scavenging and immunomodulatory effects^[13]. Because zinc has many beneficial effects on viral eradication, we performed a preliminary study which showed the beneficial effect of IFN combined with zinc supplementation on chronic hepatitis C^[14,15]. Here we present the results of a randomized controlled study of IFN with or without zinc supplementation for the treatment of chronic hepatitis C.

MATERIALS AND METHODS

Patients

Adult patients of both genders aged 24 - 70 years with

compensated chronic HCV infection were chosen. Each of the recruited patients fulfilled the following inclusion criteria: genotype 1b, more than 10^5 copies/mL of HCV in the serum, elevated serum alanine aminotransferase (ALT) concentration for at least 6 mo before initiation of treatment, and a liver biopsy specimen taken in the preceding 6 mo of study entry showing chronic hepatitis. Patients were excluded when they suffered from any of the following conditions: decompensated liver disease, other causes of liver disease such as hepatitis B infection, autoimmune disorders, hemoglobin value < 11 g/dL, white blood cell count $< 3/nL$, thrombocytopenia $< 70/nL$, neoplastic disease, severe cardiac disease, other severe concurrent diseases such as preexisting psychiatric conditions, and pregnancy or lactation. Informed consent was obtained from all patients enrolled in the study after a thorough explanation of the aims, risks and benefits of this therapy.

Study design

This randomized trial was conducted between January 2001 and December 2003 at 13 hospitals in Gunma, Japan. Eligible patients were randomly assigned to two study treatment groups with a ratio of 1:1 without stratification. Patients who met the entry criteria were randomly allocated into one of the two groups. The first group (triple therapy group) received oral triple therapy of 75 mg polaprezinc twice daily (Plomac; Zeria Pharmaceutical Co., Tokyo, Japan), IFN- α -2b (Intron A; Schering-Plough, Tokyo), and 600 mg or 800 mg ribavirin per day adjusted according to body weight (600 mg for weight below 60 kg and 800 mg for weight ≥ 60 kg) for 24 wk. The second group (dual therapy group) received combination therapy of IFN- α -2b and ribavirin at doses similar to those listed above, but no zinc supplementation. Since 75 milligrams of polaprezinc contained 17 mg of zinc, the patients received 34 mg of zinc per day. All patients were intramuscularly injected with 10 million units of IFN every day for 4 wk followed by three times a week for 20 wk. Patients were followed-up for another 24 wk after the treatment, i.e., until week 48.

The response to treatment was assessed as follows. Sustained virological response (SVR) was defined as undetectable HCV RNA in serum at the end of follow-up by analysis with Ampliform HCV version 2 with a lower limit of detection of 100 copies/mL. Analyses were performed in all patients who were randomized to one of the treatment groups. All the other patients whose HCV-RNA was positive 24 wk after the treatment were classified as non-responders.

Histopathological examination of liver

In all patients, a liver biopsy was performed in the preceding 6 mo before therapy. Hepatic inflammation (grade) and fibrosis (stage) were assessed by the semiquantitative histological score proposed by Scheuer^[16] and Desmet *et al*^[17]. Portal/periportal inflammatory activity, lobular inflammatory activity and degenerative liver cell changes were scored using a scale of 0 to 3 for the criterion of inflammatory activity (1: mild, 2: moderate, 3: severe). The degree of fibrosis was scored using a scale of 0 to 4 (0: absent, 1: mild without septa, 2: moderate with a few septa, 3: numerous septa without cirrhosis, 4: cirrhosis).

Table 1 Comparison of clinical data of patients with chronic hepatitis C who received dual or triple therapy

| | Triple therapy group | Dual therapy group | P value |
|---------------------------------------|----------------------|--------------------|--------------------|
| Demography | | | |
| Male/Female | 24/26 | 38/14 | 0.015 ¹ |
| Age ^a (yr) | 57±23 (27-69) | 53.8±9.8 (23-70) | NS ² |
| Body weight ^a (kg) | 60.5±9.8 (35-93) | 63.5±12.4 (35-93) | NS ² |
| BMI ^a (kg/m ²) | 23.5±3.1 | 23.4±2.1 | NS ² |
| Naïve/Retreatment | 34/15 | 40/11 | NS ¹ |
| Biochemistry | | | |
| HCV RNA (kcopy/mL) | 700 (100-1490) | 720 (110-2310) | NS ² |
| ALT ^a (U/L) | 95.6±61.1 | 97.4±59.8 | NS ² |
| Ferritin ^a | 216.8±244.4 | 214±139.6 | NS ² |
| Serum zinc ^a (µg/dL) | 73.3±20.3 | 69.8±17.2 | NS ² |
| Histopathology | | | |
| F 0/1/2/3/4 | 3/12/23/10/1 | 1/20/15/13/1 | NS ¹ |
| A 1/2/3 | 26/20/2 | 20/24/5 | NS ¹ |

^aData are mean \pm SE and (range).

¹ Fisher's exact test. ² Wilcoxon

NS, not significant.

Statistical analysis

Chi-square test and Fisher's exact test (two-tailed) for frequency tables were used for statistical analysis. A logistic multiple regression model was used to examine relationships between baseline clinical characteristics and the binary outcome of response to therapy. Each variable was transformed into categorical data consisting of two simple ordinal numbers for the logistic multiple regression model. Variable selection was an important step in characterizing prognostic relations and identifying variables most strongly related to the outcome. The final model was then obtained by fitting only the main effect terms. $P < 0.05$ (two-sided) was considered statistically significant. Statistical analyses were conducted by the StatView 5.0 and SAS version 8.2 statistical analysis packages.

RESULTS

Response to treatment

We recruited 102 patients with genotype 1b and more than 100 KIU/mL of HCV-RNA who met the predefined inclusion and exclusion criteria. The clinical background of the two treatment groups was not different except for gender (Table 1).

Twenty-four patients dropped out because of side effects and inability of follow-up due to unknown reasons. Thus, 78 patients including 39 patients on triple therapy and 39 who did not receive zinc supplementation were the subjects of this study (Figure 1). At the end of the study (48 wk), the SVR rate was 33.3% (13/39) in both the dual therapy and triple therapy groups (Figure 2). One week after the beginning of treatment, the viral disappearance rate in the triple therapy group (20.0% [5/25]) was not significantly different from that in the dual therapy group (10.3% [3/29]). Four weeks after the beginning of treatment, the viral disappearance rate in the triple therapy group was 69.7% (23/33), which was not significantly different from that in the dual therapy group (57.1% [16/28]).

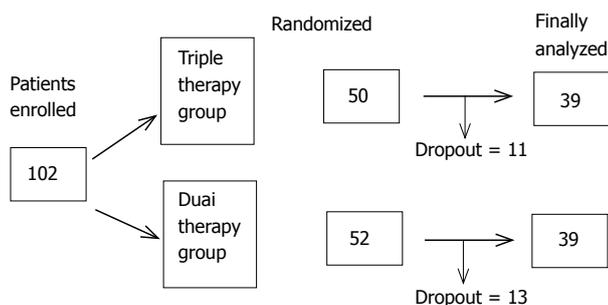


Figure 1 Profile of enrolled patients.

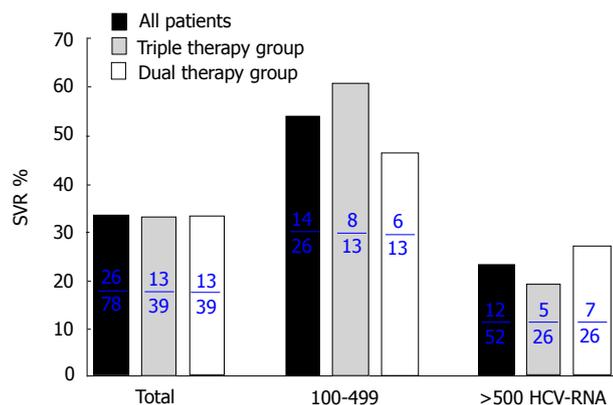


Figure 2 Rates of sustained virological response (SVR) to dual and triple therapies. Zinc supplementation had no significant effect on SVR.

Table 2 Virological response rates for dual and triple therapy groups at different weeks (w) after commencement of each treatment

| HCV RNA (kcopy/mL) | Triple therapy group | | | | Dual therapy group | | | |
|--------------------|----------------------|----------------|----------------|----------------|--------------------|----------------|----------------|----------------|
| | 1wk | 4wk | 24wk | 48wk | 1wk | 4wk | 24wk | 48wk |
| >500 | 1/17 5.9% | 13/21 61.9% | 21/26 69.2% | 5/26 19.2% | 1/20 5.0% | 10/20 50.0% | 25/26 96.2% | 7/26 26.9% |
| | | | | | NS | NS | NS | NS |
| 100-499 | 4/8 50.0% | 10/12 83.3% | 13/13 100% | 8/13 61.5% | 2/9 22.2% | 6/8 75.0% | 13/13 100% | 6/13 46.2% |
| | | | | | NS | NS | NS | NS |
| All patients | 5/25 20.0% | 23/33 69.7% | 34/39 87.2% | 13/39 33.3% | 3/29 10.3% | 16/28 57.1% | 38/39 97.4% | 13/39 33.3% |
| | | | | | NS | NS | NS | NS |

Fisher's exact test. NS, not significant.

While there were no significant differences between the two groups, patients of the triple therapy group tended to show an earlier decrease of the viral load compared to the dual therapy group (Table 2).

With regard to HCV-RNA load in the serum, the SVR rate of patients with HCV-RNA between 100 and 499 KIU/mL was 64.3% (8/13) in the triple therapy group, which was not different from that in the dual therapy group (46.2% [6/13]). Similarly, the SVR rate of patients with HCV-RNA >500 KIU/mL was not significantly different between the triple therapy and dual therapy groups

Table 3 The logistic multiple regression model

| Variable | Odds ratio | 95%CI | P-value |
|---|------------|--------------|---------|
| HCV RNA (kcopy/mL) | | | |
| <500 | 1.000 | 0.014-0.690 | 0.0197 |
| ≥500 | 0.097 | | |
| Body weight (kg) | | | |
| <64 | 1.000 | 0.931-63.301 | 0.0583 |
| ≥64 | 7.677 | | |
| Pre-treatment platelet count (×10 ⁴ /μL) | | | |
| <16 | 1.000 | 0.032-1.909 | 0.1798 |
| ≥16 | 0.246 | | |

CI: confidence interval

Table 4 Summary of adverse events n (%)

| | Triple therapy group | Dual therapy group | |
|----------------------|----------------------|--------------------|---------|
| Fever | 35 (89.7) | 38 (97.4) | NS |
| Anorexia | 26 (66.7) | 25 (64.1) | NS |
| Fatigue | 24 (61.5) | 22 (59.0) | NS |
| Arthralgia | 20 (51.3) | 18 (46.1) | NS |
| Eczema | 16 (41.0) | 13 (33.3) | NS |
| Nausea | 14 (35.9) | 13 (33.3) | NS |
| Abdominal discomfort | 8 (20.5) | 18 (46.2) | P<0.019 |
| Stomatitis | 13 (33.3) | 12 (30.8) | NS |
| Headache | 11 (28.2) | 14 (35.9) | NS |
| Hair loss | 12 (30.8) | 7 (17.9) | NS |
| Psychosomatic | 3 (7.7) | 1 (2.6) | NS |

Fisher's exact test. NS, not significant.

(5/26 [19.2%] and 7/26 [26.9%], respectively).

Factors associated with sustained virological response

A logistic multiple regression model was used for statistical analysis including HCV-RNA, body weight and pre-treatment platelet count. The analysis identified HCV-RNA level as a significant determinant of SVR when data of both groups were pooled together (Table 3).

Adverse effects

The side effects of the treatment were almost the same in both groups, including flu-like symptoms, mild reduction in hemoglobin, mild thrombocytopenia and leukopenia (Tables 4 and 5). The number of dropouts due to adverse effects was similar in the two groups (11 of 50 in the zinc group and 13 of 52 in the control group). The main reasons for dropout were anemia, fatigue, appetite loss and psychosomatic complaints (Table 6). The treatment protocol did not correlate significantly with the number of cases with adverse effects listed in Tables 4 - 6 except for abdominal discomfort. Fewer patients of the triple therapy group developed this side effect than those of the other group (P=0.019, Table 4).

DISCUSSION

The metabolism of trace elements is distorted in chronic

Table 5 Laboratory abnormalities

| | Triple therapy group | | Dual therapy group | |
|--|----------------------|----|--------------------|--|
| Decrease in WBC (<2 000 μ L) | 5 | 5 | NS | |
| (<1 500 μ L) | 1 | 0 | NS | |
| Decrease in hemoglobin (> 10 g/dL) | 13 | 14 | NS | |
| (>8.5 g/dL) | 3 | 4 | NS | |
| Decrease in platelet count (<50 000 μ L) | 1 | 3 | NS | |
| IFN dose reduction | 9 | 10 | NS | |
| Ribavirin dose reduction | 15 | 17 | NS | |

Fisher's exact test. NS, not significant.

liver disease^[6]. We have previously reported that zinc deficiency in patients with chronic HCV can be improved by treatment with IFN^[18]. In our previous pilot study, the beneficial effects of combined treatment of zinc and IFN have been confirmed with regard to both eradication of the virus and lowering or normalization of ALT^[15].

In view of the clinical background of patients before treatment, the number of female patients in our study was greater than that of male patients in the triple therapy group, but univariate analysis did not reveal a significant sex difference in the effect of treatment.

Although patients on triple therapy showed early disappearance of HCV-RNA, the results 24 wk after the treatment showed no overall beneficial effect of zinc supplementation and are similar to those of previous reports^[15]. Furthermore, the triple therapy tended to have the same beneficial effects of IFN + ribavirin dual therapy in patients with moderate viral load (between 100 and 499 KIU/mL). It is possible that longer treatment (more than 24 wk) and perhaps higher dosages of IFN and ribavirin may produce different results. Based on the adverse effects of the treatment, the only advantage of the triple therapy is the significantly lower incidence of abdominal discomfort compared to the dual therapy.

Polaprezinc, a synthetic agent, N-(3-aminopropionyl)-L-histidinate zinc, is a chelating compound for ulcer healing and has been used experimentally to prevent gastric ulceration in rats as well as clinically as an anti-peptic ulcer drug^[19,20]. Zinc present in the orally administered polaprezinc is absorbed, leading to a rise in serum concentration of zinc^[21]. In this respect, absorption of polaprezinc is more favorable than other zinc-containing derivatives such as zinc sulfate^[21]. While the dose of polaprezinc could increase serum zinc concentration and possibly improve the SVR, caution should be exercised with regard to the side effects of higher doses of polaprezinc, as it is known to induce abdominal discomfort in some patients (personal communication).

In conclusion, 24-wk zinc supplementation has no additive effect on the anti-HCV dual therapy of IFN- α -2 and ribavirin, apart from reducing the incidence of abdominal discomfort.

ACKNOWLEDGMENTS

This study was performed by Gunma Liver Study Group, Japan.

Table 6 Reasons for withdrawal from the study

| Causes | Triple therapy group | | Dual therapy group | |
|--------------------|----------------------|----|--------------------|--|
| Anemia | 2 | 3 | NS | |
| Psychosomatic | 3 | 4 | NS | |
| Anorexia & Fatigue | 2 | 3 | NS | |
| HCC ¹ | 1 | 0 | NS | |
| Dizziness | 0 | 1 | NS | |
| Lost to follow-up | 3 | 2 | NS | |
| Total | 11 | 13 | NS | |

Fisher's exact test. NS, not significant.

¹Hepatocellular carcinoma was detected during the treatment.

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